PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 22 April 1999 (22.04.99)	
International application No. PCT/EP98/04810	**Applicant's or agent's file reference** C 1974 PCT
International filing date (day/month/year) 31 July 1998 (31.07.98)	**Priority date** (day/month/year) 01 August 1997 (01.08.97)
Applicant WANKER, Erich et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

26 February 1999 (26.02.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer F. Gateau
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

VERTRAG [redacted]R DIE INTERNATIONALE ZUS[redacted]ENARBEIT
[redacted] DEM GEBIET DES PATENTWESENS

Absender: INTERNATIONALE RECHERCHENBEHÖRDE

PCT

MITTEILUNG ÜBER DIE ÜBERMITTLUNG DES INTERNATIONALEN RECHERCHENBERICHTS ODER DER ERKLÄRUNG

(Regel 44.1 PCT)

An
VOSSIUS & PARTNER
Postfach 86 07 67
D-81634 München
GERMANY

EINGEGANGEN
Vossius & Partner
21. Mai 1999

Frist bearb.: 18.7. 18.8.99

Aktenzeichen des Anmelders oder Anwalts C 1974 PCT	Absendedatum (Tag/Monat/Jahr) 18/05/1999
Internationales Aktenzeichen PCT/EP 98/04810	**WEITERES VORGEHEN** siehe Punkte 1 und 4 unten
Anmelder MAX-PLANCK-GESELLSCHAFT ZUR FORDERUNG DER et al.	Internationales Anmeldedatum (Tag/Monat/Jahr) 31/07/1998

1. [X] Dem Anmelder wird mitgeteilt, daß der internationale Recherchenbericht erstellt wurde und ihm hiermit übermittelt wird.

Einreichung von Änderungen und einer Erklärung nach Artikel 19:
Der Anmelder kann auf eigenen Wunsch die Ansprüche der internationalen Anmeldung ändern (siehe Regel 46):

Bis wann sind Änderungen einzureichen?

Die Frist zur Einreichung solcher Änderungen beträgt üblicherweise zwei Monate ab der Übermittlung des internationalen Recherchenberichts; weitere Einzelheiten sind den Anmerkungen auf dem Beiblatt zu entnehmen.

Wo sind Änderungen einzureichen?

Unmittelbar beim Internationalen Büro der WIPO, 34, CHEMIN des Colombettes, CH-1211 Genf 20, Telefaxnr.: (41-22) 740.14.35

Nähere Hinweise sind den Anmerkungen auf dem Beiblatt zu entnehmen.

2. [ ] Dem Anmelder wird mitgeteilt, daß kein internationaler Recherchenbericht erstellt wird und daß ihm hiermit die Erklärung nach Artikel 17(2)a) übermittelt wird.

3. [ ] **Hinsichtlich des Widerspruchs** gegen die Entrichtung einer zusätzlichen Gebühr (zusätzlicher Gebühren) nach Regel 40.2 wird dem Anmelder mitgeteilt, daß

[ ] der Widerspruch und die Entscheidung hierüber zusammen mit seinem Antrag auf Übermittlung des Wortlauts sowohl des Widerspruchs als auch der Entscheidung hierüber an die Bestimmungsämter dem Internationalen Büro übermittelt worden sind.

[ ] noch keine Entscheidung über den Widerspruch vorliegt; der Anmelder wird benachrichtigt, sobald eine Entscheidung getroffen wurde.

4. **Weiteres Vorgehen:** Der Anmelder wird auf folgendes aufmerksam gemacht:

Kurz nach Ablauf von **18 Monaten** seit dem Prioritätsdatum wird die internationale Anmeldung vom Internationalen Büro veröffentlicht. Will der Anmelder die Veröffentlichung verhindern oder auf einen späteren Zeitpunkt verschieben, so muß gemäß Regel 90 bis.1 bzw. 90 bis.3 vor Abschluß der technischen Vorbereitungen für die internationale Veröffentlichung eine Erklärung über die Zurücknahme der internationalen Anmeldung oder des Prioritätsanspruchs beim Internationalen Büro eingehen.

Innerhalb von **19 Monaten** seit dem Prioritätsdatum ist ein Antrag auf internationale vorläufige Prüfung einzureichen, wenn der Anmelder den Eintritt in die nationale Phase bis zu 30 Monaten seit dem Prioritätsdatum (in manchen Ämtern sogar noch länger) verschieben möchte.

Innerhalb von **20 Monaten** seit dem Prioritätsdatum muß der Anmelder die für den Eintritt in die nationale Phase vorgeschriebenen Handlungen vor allen Bestimmungsämtern vornehmen, die nicht innerhalb von 19 Monaten seit dem Prioritätsdatum in der Anmeldung oder einer nachträglichen Auswahlerklärung ausgewählt wurden oder nicht ausgewählt werden konnten, da für sie Kapitel II des Vertrages nicht verbindlich ist.

Name und Postanschrift der Internationalen Recherchenbehörde Europäisches Patentamt, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Bevollmächtigter Bediensteter Jaap Hurenkamp

(Siehe Anmerkungen auf Beiblatt)

ANMERKUNGEN ZU FORMBLATT PCT/ISA/220

Diese Anmerkungen sollen grundlegende Hinweise zur Einreichung von Änderungen gemäß Artikel 19 geben. Diesen Anmerkungen liegen die Erfordernisse des Vertrags über die internationale Zusammenarbeit auf dem Gebiet des Patentwesens (PCT), der Ausführungsordnung und der Verwaltungsrichtlinien zu diesem Vertrag zugrunde. Bei Abweichungen zwischen diesen Anmerkungen und obengenannten Texten sind letztere maßgebend. Nähere Einzelheiten sind dem PCT-Leitfaden für Anmelder, einer Veröffentlichung der WIPO, zu entnehmen.
Die in diesen Anmerkungen verwendeten Begriffe "Artikel", "Regel" und "Abschnitt" beziehen sich jeweils auf die Bestimmungen des PCT-Vertrags, der PCT-Ausführungsordnung bzw. der PCT-Verwaltungsrichtlinien.

HINWEISE ZU ÄNDERUNGEN GEMÄSS ARTIKEL 19

Nach Erhalt des internationalen Recherchenberichts hat der Anmelder die Möglichkeit, einmal die Ansprüche der internationalen Anmeldung zu ändern. Es ist jedoch zu betonen, daß, da alle Teile der internationalen Anmeldung (Ansprüche, Beschreibung und Zeichnungen) während des internationalen vorläufigen Prüfungsverfahrens geändert werden können, normalerweise keine Notwendigkeit besteht, Änderungen der Ansprüche nach Artikel 19 einzureichen, außer wenn der Anmelder z.B. zum Zwecke eines vorläufigen Schutzes die Veröffentlichung dieser Anspruche wünscht oder ein anderer Grund für eine Änderung der Ansprüche vor ihrer internationalen Veröffentlichung vorliegt. Weiterhin ist zu beachten, daß ein vorläufiger Schutz nur in einigen Staaten erhältlich ist.

Welche Teile der internationalen Anmeldung können geändert werden?

Im Rahmen von Artikel 19 können nur die Ansprüche geändert werden.

In der internationalen Phase können die Ansprüche auch nach Artikel 34 vor der mit der internationalen vorläufigen Prüfung beauftragten Behörde geändert (oder nochmals geändert) werden. Die Beschreibung und die Zeichnungen können nur nach Artikel 34 vor der mit der internationalen vorläufigen Prüfung beauftragten Behörde geändert werden.

Beim Eintritt in die nationale Phase können alle Teile der internationalen Anmeldung nach Artikel 28 oder gegebenenfalls Artikel 41 geändert werden.

Bis wann sind Änderungen einzureichen?

Innerhalb von zwei Monaten ab der Übermittlung des internationalen Recherchenberichts oder innerhalb von sechzehn Monaten ab dem Prioritätsdatum, je nachdem, welche Frist später abläuft. Die Änderungen gelten jedoch als rechtzeitig eingereicht, wenn sie dem Internationalen Büro nach Ablauf der maßgebenden Frist, aber noch vor Abschluß der technischen Vorbereitungen für die internationale Veröffentlichung (Regel 46.1) zugehen.

Wo sind die Änderungen nicht einzureichen?

Die Änderungen können nur beim Internationalen Büro, nicht aber beim Anmeldeamt oder der Internationalen Recherchenbehörde eingereicht werden (Regel 46.2).

Falls ein Antrag auf internationale vorläufige Prüfung eingereicht wurde/wird, siehe unten.

In welcher Form können Änderungen erfolgen?

Eine Änderung kann erfolgen durch Streichung eines oder mehrerer ganzer Ansprüche, durch Hinzufügung eines oder mehrerer neuer Ansprüche oder durch Änderung des Wortlauts eines oder mehrerer Ansprüche in der eingereichten Fassung.

Für jedes Anspruchsblatt, das sich aufgrund einer oder mehrerer Änderungen von dem ursprünglich eingereichten Blatt unterscheidet, ist ein Ersatzblatt einzureichen.

Alle Ansprüche, die auf einem Ersatzblatt erscheinen, sind mit arabischen Ziffern zu numerieren. Wird ein Anspruch gestrichen, so brauchen, die anderen Ansprüche nicht neu numeriert zu werden. Im Fall einer Neunumerierung sind die Ansprüche fortlaufend zu numerieren (Verwaltungsrichtlinien, Abschnitt 205 b)).

Die Änderungen sind in der Sprache abzufassen, in der dieinternationale Anmeldung veröffentlicht wird.

Welche Unterlagen sind den Änderungen beizufügen?

Begleitschreiben (Abschnitt 205 b)):

Die Änderungen sind mit einem Begleitschreiben einzureichen.

Das Begleitschreiben wird nicht zusammen mit der internationalen Anmeldung und den geänderten Ansprüchen veröffentlicht. Es ist nicht zu verwechseln mit der "Erklärung nach Artikel 19(1)" (siehe unten, "Erklärung nach Artikel 19 (1)").

Das Begleitschreiben ist nach Wahl des Anmelders in englischer oder französischer Sprache abzufassen. Bei englischsprachigen internationalen Anmeldungen ist das Begleitschreiben aber ebenfalls in englischer, bei französischsprachigen internationalen Anmeldungen in französischer Sprache abzufassen.

ANMERKUNGEN ZU FORMBLATT PCT/ISA/220 (Fortsetzung)

Im Begleitschreiben sind die Unterschiede zwischen den Ansprüchen in der eingereichten Fassung und den geänderten Ansprüchen anzugeben. So ist insbesondere zu jedem Anspruch in der internationalen Anmeldung anzugeben (gleichlautende Angaben zu verschiedenen Ansprüchen können zusammengefaßt werden), ob

i) der Anspruch unverändert ist;

ii) der Anspruch gestrichen worden ist;

iii) der Anspruch neu ist;

iv) der Anspruch einen oder mehrere Ansprüche in der eingereichten Fassung ersetzt;

v) der Anspruch auf die Teilung eines Anspruchs in der eingereichten Fassung zurückzuführen ist.

Im folgenden sind Beispiele angegeben, wie Änderungen im Begleitschreiben zu erläutern sind:

1. [Wenn anstelle von ursprünglich 48 Ansprüchen nach der Änderung einiger Ansprüche 51 Ansprüche existieren]:
"Die Ansprüche 1 bis 29, 31, 32, 34, 35, 37 bis 48 werden durch geänderte Ansprüche gleicher Numerierung ersetzt; Ansprüche 30, 33 und 36 unverändert; neue Ansprüche 49 bis 51 hinzugefügt."

2. [Wenn anstelle von ursprünglich 15 Ansprüchen nach der Änderung aller Ansprüche 11 Ansprüche existieren]:
"Geänderte Ansprüche 1 bis 11 treten an die Stelle der Ansprüche 1 bis 15."

3. [Wenn ursprünglich 14 Ansprüche existierten und die Änderungen darin bestehen, daß einige Ansprüche gestrichen werden und neue Ansprüche hinzugefügt werden]:
Ansprüche 1 bis 6 und 14 unverändert; Ansprüche 7 bis 13 gestrichen; neue Ansprüche 15, 16 und 17 hinzugefügt."Oder" Ansprüche 7 bis 13 gestrichen; neue Ansprüche 15, 16 und 17 hinzugefügt; alle übrigen Ansprüche unverändert."

4. [Wenn verschiedene Arten von Änderungen durchgeführt werden]:
"Ansprüche 1-10 unverändert; Ansprüche 11 bis 13, 18 und 19 gestrichen; Ansprüche 14, 15 und 16 durch geänderten Anspruch 14 ersetzt; Anspruch 17 in geänderte Ansprüche 15, 16 und 17 unterteilt; neue Ansprüche 20 und 21 hinzugefügt."

"Erklärung nach Artikel 19(1)" (Regel 46.4)

Den Änderungen kann eine Erklärung beigefügt werden, mit der die Änderungen erläutert und ihre Auswirkungen auf die Beschreibung und die Zeichnungen dargelegt werden (die nicht nach Artikel 19 (1) geändert werden können).

Die Erklärung wird zusammen mit der internationalen Anmeldung und den geänderten Ansprüchen veröffentlicht.

Sie ist in der Sprache abzufassen, in der die internationale Anmeldung veröffentlicht wird.

Sie muß kurz gehalten sein und darf, wenn in englischer Sprache abgefaßt oder ins Englische übersetzt, nicht mehr als 500 Wörter umfassen

Die Erklärung ist nicht zu verwechseln mit dem Begleitschreiben, das auf die Unterschiede zwischen den Ansprüchen in der eingereichten Fassung und den geänderten Ansprüchen hinweist, und ersetzt letzteres nicht. Sie ist auf einem gesonderten Blatt einzureichen und in der Überschrift als solche zu kennzeichnen, vorzugsweise mit den Worten "Erklärung nach Artikel 19 (1)".

Die Erklärung darf keine herabsetzenden Äußerungen über den internationalen Recherchenbericht oder die Bedeutung von in dem Bericht angeführten Veröffentlichungen enthalten. Sie darf auf im internationalen Recherchenbericht angeführte Veröffentlichungen, die sich auf einen bestimmten Anspruch beziehen, nur im Zusammenhang mit einer Änderung dieses Anspruchs Bezug nehmen.

Auswirkungen eines bereits gestellten Antrags auf internationale vorläufige Prüfung

Ist zum Zeitpunkt der Einreichung von Änderungen nach Artikel 19 bereits ein Antrag auf internationale vorläufige Prüfung gestellt worden, so sollte der Anmelder in seinem Interesse gleichzeitig mit der Einreichung der Änderungen beim Internationalen Büro auch eine Kopie der Änderungen bei der mit der internationalen vorläufigen Prüfung beauftragen Behörde einreichen (siehe Regel 62.2 a), erster Satz).

Auswirkungen von Änderungen hinsichtlich der Übersetzung der internationalen Anmeldung beim Eintritt in die nationale Phase

Der Anmelder wird darauf hingewiesen, daß bei Eintritt in die nationale Phase möglicherweise anstatt oder zusätzlich zu der Übersetzung der Ansprüche in der eingereichten Fassung eine Übersetzung der nach Artikel 19 geänderten Ansprüche an die bestimmten/ausgewählten Ämter zu übermitteln ist.

Nähere Einzelheiten über die Erfordernisse jedes bestimmten/ausgewählten Amts sind Band II des PCT-Leitfadens für Anmelder zu entnehmen.

PATENT COOPERATION TREATY

RECEIVED 2 NOV 1999
WIPO PCT

4

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
C 1974 PCT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP98/04810	31/07/1998	01/08/1997
International Patent Classification (IPC) or national classification and IPC G01N33/60		
Applicant MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG ... et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
26/02/1999	29. 10. 99
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Wieser, M Telephone No. +49 89 2399 8434

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP98/04810

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-38 as originally filed

Claims, No.:

1-25 as originally filed

Drawings, sheets:

1/12-12/12 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 21-25.

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. 21-25 are so unclear that no meaningful opinion could be formed (*specify*):

see separate sheet

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	4,6-9,11,13-20
	No:	Claims	1-3,5,10,12
Inventive step (IS)	Yes:	Claims	13-17
	No:	Claims	1-12,18-20
Industrial applicability (IA)	Yes:	Claims	1-20
	No:	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Section III:

The chemical products according to claims 21-25 are not defined in a way which allows to unambiguously identify them. These claims are not clear and do not meet the requirements of Article 6 PCT. A meaningful examination of novelty and inventive step is not possible.

Section V:

1. An amyloid is a fibrous glycoprotein, which at specific diseases (amyloidases) is extracelullar deposited in various organs of the body. In general it consists of unbranched filamentous fibrils, which are about 10 nm thin and up to 1 µm long.

   EP-A-0 206 302 teaches a method for detection of serum amyloid A protein (SAA) or serum amyloid P-component (SAP), wherein a carrier, like a filter paper coated with nitrated phenyl groups, is contacted with a sample containing said proteins, which were bound to said carrier and thereafter immunologically detected. In the case of SAP, calcium ions have to be present (see claims and pages 3-4).

   The disclosure in document A anticipates the subject-matter of claims 1-3,5,10 and 12. These claims are not novel and do not meet the requirements of Article 33(2) PCT.

   The subject-matter of claims 4, 6-9,11 and 18-20 (as far as they refer to claims 1-12) is not based on an inventive concept per se, contrary to the requirements of Article 33(3) PCT.

2. The subject-matter of claims 13-17, and of claims 18-20 (as far as they refer to claims 13-17), is not disclosed in the documents cited in the International Search report and cannot be derived from said documents in an obvious way, either alone or in any combination. These claims seem to meet the requirements of Articles 3(2) and 33(3) PCT.

Section VIII:

1. The subject-matter of independent claim 1 is not clear (Article 6 PCT).

   Article 6 PCT has to be interpreted as meaning not only that an independent claim must be comprehensible from a technical point of view but also that it must define clearly the object of the invention, that is to say indicate all the essential features thereof.

   At present claim 1 does not give any technical feature that would allow a skilled person to carry out the claimed detection method. The claim refers to a method for detecting specific substances comprising

   (a) contacting a filter with the substances, and

   (b) detecting whether the substances are on the filter.

   This definition of the invention does not contain any technical information (like the nature of the filter or the method of detection) and is merely a description of the result to be achieved. The information that the substances to be detected are indicative of specific diseases (claims 2-5), is unable to define a method of detection.

2. Besides the objection raised in Section III above, the subject-matter of claims 21-25, does not seem to be so linked with the subject-matter of claims 1-20 as to form a single general inventive concept (Rule 13.1 PCT).

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

VOSSIUS & PARTNER
Siebertstrasse 4
81675 München
ALLEMAGNE

EINGEGANGEN
Vossius & Partner
03. Nov. 1999
Frist
bearb.:

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	29. 10. 99
Applicant's or agent's file reference C 1974 PCT	IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP98/04810	31/07/1998	01/08/1997

Applicant
MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG ... et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Digiusto, M Tel. +49 89 2399-8162

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
C 1974 PCT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP98/04810	31/07/1998	01/08/1997
International Patent Classification (IPC) or national classification and IPC		
G01N33/60		
Applicant		
MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG ... et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
26/02/1999	29. 10. 99
Name and mailing address of the international preliminary examining authority:	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Wieser, M Telephone No. +49 89 2399 8434

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP98/04810

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-38 as originally filed

Claims, No.:

1-25 as originally filed

Drawings, sheets:

1/12-12/12 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 21-25.

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. 21-25 are so unclear that no meaningful opinion could be formed (*specify*):

see separate sheet

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	4,6-9,11,13-20
	No:	Claims	1-3,5,10,12
Inventive step (IS)	Yes:	Claims	13-17
	No:	Claims	1-12,18-20
Industrial applicability (IA)	Yes:	Claims	1-20
	No:	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Section III:

The chemical products according to claims 21-25 are not defined in a way which allows to unambiguously identify them. These claims are not clear and do not meet the requirements of Article 6 PCT. A meaningful examination of novelty and inventive step is not possible.

Section V:

1. An amyloid is a fibrous glycoprotein, which at specific diseases (amyloidases) is extracelullar deposited in various organs of the body. In general it consists of unbranched filamentous fibrils, which are about 10 nm thin and up to 1 µm long.

   EP-A-0 206 302 teaches a method for detection of serum amyloid A protein (SAA) or serum amyloid P-component (SAP), wherein a carrier, like a filter paper coated with nitrated phenyl groups, is contacted with a sample containing said proteins, which were bound to said carrier and thereafter immunologically detected. In the case of SAP, calcium ions have to be present (see claims and pages 3-4).

   The disclosure in document A anticipates the subject-matter of claims 1-3,5,10 and 12. These claims are not novel and do not meet the requirements of Article 33(2) PCT.

   The subject-matter of claims 4, 6-9,11 and 18-20 (as far as they refer to claims 1-12) is not based on an inventive concept per se, contrary to the requirements of Article 33(3) PCT.

2. The subject-matter of claims 13-17, and of claims 18-20 (as far as they refer to claims 13-17), is not disclosed in the documents cited in the International Search report and cannot be derived from said documents in an obvious way, either alone or in any combination. These claims seem to meet the requirements of Articles 3(2) and 33(3) PCT.

Section VIII:

1. The subject-matter of independent claim 1 is not clear (Article 6 PCT).

   Article 6 PCT has to be interpreted as meaning not only that an independent claim must be comprehensible from a technical point of view but also that it must define clearly the object of the invention, that is to say indicate all the essential features thereof.

   At present claim 1 does not give any technical feature that would allow a skilled person to carry out the claimed detection method. The claim refers to a method for detecting specific substances comprising

   (a) contacting a filter with the substances, and

   (b) detecting whether the substances are on the filter.

   This definition of the invention does not contain any technical information (like the nature of the filter or the method of detection) and is merely a description of the result to be achieved. The information that the substances to be detected are indicative of specific diseases (claims 2-5), is unable to define a method of detection.

2. Besides the objection raised in Section III above, the subject-matter of claims 21-25, does not seem to be so linked with the subject-matter of claims 1-20 as to form a single general inventive concept (Rule 13.1 PCT).

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classificati n 6 : G01N 33/60

A2

(11) International Publication Number: **WO 99/06838**

(43) International Publication Date: 11 February 1999 (11.02.99)

(21) International Application Number: PCT/EP98/04810

(22) International Filing Date: 31 July 1998 (31.07.98)

(30) Priority Data: 97113320.2 1 August 1997 (01.08.97) EP

(71) Applicant *(for all designated States except US)*: MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V. [DE/DE]; Berlin (DE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: WANKER, Erich [AT/DE]; Leichhardtstrasse 61, D-14195 Berlin (DE). LEHRACH, Hans [AT/DE]; Lützelsteiner Weg 50, D-14195 Berlin (DE). SCHERZINGER, Eberhard [DE/DE]; Lützelsteiner Weg 52, D-14195 Berlin (DE). BATES, Gillian [GB/GB]; United Medical and Dental Schools, Division of Medical and Molecular Genetics, Guy's Tower, 8th floor, Guy's Hospital, London SE1 9RT (GB).

(74) Agent: VOSSIUS & PARTNER; Postfach 86 07 67, D-81634 München (DE).

(81) Designated States: CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published

Without international search report and to be republished upon receipt of that report.

(54) Title: NOVEL METHOD OF DETECTING AMYLOID-LIKE FIBRILS OR PROTEIN AGGREGATES

(57) Abstract

The present invention relates to methods of detecting the presence of detergent- or urea-insoluble amyloid-like fibrils or protein aggregates on filters. Preferably, said fibrils or aggregates are indicative of a disease, preferably of a neurodegenerative disease such as Alzheimer's disease or Huntington's disease. In addition, the present invention relates to inhibitors identified by the method of the invention, to pharmaceutical compositions comprising said inhibitors and to diagnostic compositions useful for the investigation of said amyloid-like fibrils or aggregates.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

Novel method of detecting amyloid-like fibrils or protein aggregates

The present invention relates to methods of detecting the presence of detergent- or urea-insoluble amyloid-like fibrils or protein aggregates on filters. Preferably, said fibrils or aggregates are indicative of a disease, preferably of a neurodegenerative disease such as Alzheimer's disease or Huntington's disease. In addition, the present invention relates to inhibitors identified by the method of the invention, to pharmaceutical compositions comprising said inhibitors and to diagnostic compositions useful for the investigation of said amyloid-like fibrils or aggregates.

Amyloid-like fibrils and aggregates are found widespread in nature. For example, protein aggregates are found as inclusion bodies in bacteria. Such inclusion bodies may in particular arise during the recombinant expression of proteins in bacteria. In addition, a variety of diseases, both in humans and animals, is characterized by the pathogenic formation of amyloid-like fibrils or protein aggregates in neuronal tissues. A well-known and typical example of such diseases is Alzheimer's disease (AD). AD is characterized by the formation of neurofibrillar tangles and β-amyloid fibrils in the brain of AD patients. Similarly, scrapie is associated with the occurrence of scrapie-associated fibrils in brain tissue.

Another class of these diseases is characterized by an expansion of CAG repeats in certain genes. The affected proteins display a corresponding polyglutamine expansion. Said diseases are further characterized by a late onset in life and a dominant pathway of inheritance.

A typical representative of this class of diseases is Huntington's disease. Huntington's disease (HD) is an autosomal dominant progressive neurodegenerative disorder characterized by personality changes, motor impairment and subcortical dementia (Harper, 1991). It is associated with a selective neuronal cell death occurring primarily in the cortex and striatum (Vonsattel et al., 1985). The disorder is caused by a CAG/polyglutamine (polygln)

repeat expansion in the first exon of a gene encoding a large ~350 kDa protein of unknown function, designated huntingtin (HDCRG, 1993). The CAG repeat is highly polymorphic and varies from 6-39 repeats on chromosomes of unaffected individuals and 35-180 repeats on HD chromosomes (Rubinsztein et al., 1996; Sathasivam et al., 1997). The majority of adult onset cases have expansions ranging from 40-55 units, whereas expansions of 70 and above invariably cause the juvenile form of the disease. The normal and mutant forms of huntingtin have been shown to be expressed at similar levels in the central nervous system and in peripheral tissues (Trottier et al., 1995a). Within the brain, huntingtin was found predominantly in neurons and was present in cell bodies, dentrites and also in the nerve terminals. Immunohistochemistry, electron microscopy and subcellular fractionations have shown that huntingtin is primarily a cytosolic protein associated with vesicles and/or microtubules, suggesting that it plays a functional role in cytoskeletal anchoring or transport of vesicles (DiFiglia et al., 1995; Gutekunst et al., 1995; Sharp et al., 1995) Huntingtin has also been detected in the nucleus (de Rooij et al., 1996; Hoogeveen et al., 1993) suggesting that transcriptional regulation cannot be ruled out as a possible function of this protein.

In addition to HD, CAG/polygln expansions have been found in at least six other inherited neurodegenerative disorders which include: spinal and bulbar muscular atrophy (SBMA), dentatorubral pallidoluysian atrophy (DRPLA), and the spinocerebellar ataxias (SCA) types 1, 2, 3 and 6 (referenced in Bates et al. 1997). The normal and expanded size ranges are comparable with the exception of SCA6 in which the expanded alleles are smaller and the mutation is likely to act by a different route. However, in all cases the CAG repeat is located within the coding region and is translated into a stretch of polygln residues. Although the proteins harboring the polygln sequences are unrelated and mostly of unknown function, it is likely that the mutations act through a similar mechanism. Without exception, these proteins are widely expressed and generally localized in the cytoplasm. However, despite overlapping expression patterns in brain, the neuronal cell death is relatively specific and can differ markedly (Ross, 1995), indicating that additional factors are needed to convey the specific patterns of neurodegeneration.

In the art, there is a variety of methods to determine the presence of amyloid-like fibrils or protein aggregates. For example, inclusion bodies in bacteria can be made visible microscopically. Further, amyloid-like fibrils such as from Alzheimer's

disease may be analyzed by complex methodology; see, for example, Booth et al. Nature 385 (1997), 787-793, and references cited therein.

So far, however, a simple method that may be established in any laboratory without sophisticated equipment is not available. Such a method would be especially useful in routine procedures such as the testing of patient samples for amyloid-like fibrils or protein aggregates. Accordingly, the technical problem underlying the present invention was to provide a rather simple method that may routinely be used for the detection of such fibrils or aggregates. The solution to said problem is provided by the embodiments characterized in the claims.

Thus, the present invention relates to a method of detecting the presence of detergent- or urea-insoluble amyloid-like fibrils or protein aggregates on a filter comprising the steps of (a) contacting said filter with material suspected to comprise said fibrils or aggregates and (b) detecting whether said fibrils or aggregates are retained on said filter.

In accordance with the present invention, it has surprisingly been found that filters of a variety of consistencies may be employed to retain detergent- or urea-insoluble amyloid-like fibrils or protein aggregates on their surface. Essentially, only the above-recited two steps are necessary in order to investigate whether said fibrils or aggregates are present in a sample. The first step comprises contacting the filter with material suspected to comprise said fibrils or aggregates. The term "suspected to comprise" is intended to mean that the investigator may start from the assumption that the material indeed contains such fibrils or aggregates. Alternatively, said term means that it is totally unclear whether the material under investigation comprises such fibrils or aggregates.

It may be appropriate to pretreat the material prior to application to the filter. For example, for the detection of inclusion bodies, it may be necessary to first lyse the bacteria and set the cytoplasmic fraction free. Also, it may be useful to pretreat the patient samples prior to application to the filter. Said pretreatment may be effected, for example by employing proteases.

The detection of fibrils or aggregates that are retained on the filter (the second step) may also be effected by a variety of steps. For example, detection may be effected by Western blot techniques, if an appropriate antibody is available.

In a preferred embodiment of the method of the invention, said amyloid-like fibrils or protein aggregates are indicative of a disease.
Of particular advantage is the method of the present invention in the detection of amyloid fibrils or protein aggregates that are indicative of human diseases, particularly in routine laboratory methods. This embodiment of the method of the invention allows the rapid determination of the disease state, if any, of the patient. For example, the concentration of amyloid-like fibrils or protein aggregates in tissue may be determined by appropriate dilution series. Using automated systems, the presence of such fibrils or aggregates per se may be determined for a large number of patients on a single filter. A further advantage of the method of the invention is that results are available rather quickly. This has also an impact on the overall cost of the detection method, in particular in routine laboratory diagnosis. Due to the simple set-up of the method of the invention, a large number of routine laboratories, for example in hospitals, can apply the method of the invention without the need to acquire expensive equipment such as electron microscopes.

Thus, particularly preferred is the method where said disease is a human disease.

Further preferred is that said disease is associated with a polyglutamine expansion.

Most preferred is that said disease is Huntington's disease, spinal and bulbar muscular atrophy, dentarorubral pallidoluysian atropy, spinocerebellar ataxia type-1, -2, -3, -6, or –7, Alzheimer's disease, BSE, primary systemic amyloidosis, secondary systemic amyloidosis, senile systemic amyloidosis, familial amyloid polyneuropathy I, hereditary cerebral amyloid angiopathy, hemodialysis-related amyloidosis, familial amyloid polyneuropathy III, Finnish hereditary systemic amyloidosis, type II diabetes, medullary carcinoma of the thyroid, spongiform encephalopathies: Kuru, Gerstmann- Sträussler-Scheinker syndrome (GSS), familial insomnia, scrapie, atrial amyloidosis, hereditary non-neuropathic systemic amyloidosis, injection-localized amyloidosis, hereditary renal amyloidosis, or Parkinson's disease.
In particular, these diseases, for which, as a rule, no simple detection method has been developed so far, can now be assessed by simple laboratory technology.

The filter used in the method of the invention may be any filter with a sufficiently small pore size (0.45 µm and preferably 0.2 µm or smaller). Preferred is that the filter is comprised of material with low protein absorption, and particularly preferred is that the material with low protein absorption of which said filter is comprised is cellulose acetate.

A further preferred embodiment relates to a method wherein, prior to step (b), the following step is carried out:

(b') washing said filter so as to remove detergent- or urea-soluble material.

This embodiment is particularly preferred when enhanced sensitivity of detection is required. Such an enhanced sensitivity may be necessary, for example, when the early onset of one of the above-mentioned diseases is assessed because it is envisaged that at an early stage of any of said diseases a rather low amount of fibrils or aggregates may be found in the affected tissue or cells.

Step (b') may be repeated one or several times. The person skilled in the art is in a position to determine appropriate washing conditions without further ado. Preferably, the washing buffer comprises 0.1-2% SDS, 4-8M urea, and 0.1-2% Triton X-100.

Further preferred is in the method of the invention that the detergent- or urea-soluble material is simultaneously with or subsequent to step (a), sucked through said filter.

This embodiment is particularly useful when a microtitre plate containing a filter membrane or an apparatus for dot-blotting is available. The non-desired detergent- or urea-soluble material may be easily removed applying, for example, such a dot-blot apparatus.

In another preferred embodiment of the invention, detection in step (b) is effected by an antibody, or (poly)peptide, preferably a tag or an enzyme, or a fragment or derivative thereof or a chemical reagent that specifically binds to said fibrils or aggregates.

As regards the antibody, or fragment or derivative thereof, it may be employed in the Western blot type assay to determine the presence of said fibrils or aggregates. Western blot technology is well-known in the art and need not be described here in any more detail.

In another preferred embodiment of the invention, detection in step (b) is effected by electron microscopy, electron scanning microscopy, fluorescence or chemiluminescence.

In a further preferred embodiment of the method of the invention, said material is derived from tissues or cells of bacteria, yeast, fungi, plants, insects, animals, preferably mammals, humans, from a transgenic animal or a transgenic plant.

In accordance with the present invention, it is additionally preferred that the method further comprises the following steps:

(a') incubating a fusion protein comprising a (poly)peptide that enhances solubility and/or prevents aggregation of said fusion protein, an amyloidogenic (poly)peptide that has the ability to self-assemble into amyloid-like fibrils or protein aggregates when released from said fusion protein and a cleavable site that separates the above-mentioned components of the fusion protein in the presence of a suspected inhibitor of amyloid-like fibril or protein aggregate formation; and

(a") simultaneously with or after step (a'), further incubating with a compound that induces cleavage at said cleavage site.

This embodiment is particularly advantageous for elucidating the mechanism or basis of amyloid-like fibril or protein aggregate formation. Namely, it has been surprisingly found that proteins of the above composition, after cleavage, aggregate under conditions that are, e.g., described in the appended examples. Thus, fibril or aggregate formation may be monitored under varying conditions and detected by the filter assay of the present invention.

The (poly)peptide that enhances solubility and/or prevents aggregation is preferably glutathione-S-transferase, intein, thioredoxin, dihydroflate reductase, chymotrypsin inhibitor II or a functional fragment or derivative thereof. A functional fragment is a fragment that essentially retains the function of the (poly)peptide.

Preferred proteins that have the ability of self-assembling to amyloid-like fibrils or protein aggregates may be selected from the group consisting or huntingtin, androgen receptor, atropin, TATA binding protein, or ataxin-1,-2,-3 or -6 or a fragment or derivative thereof, amyloid precursor protein (APP), ß-protein, an immunoglobulin light chain, serum amyloid A, transthyretin, cystatin C, ß2-microglobulin, apolipoprotein A-1, gelsoline, islet amyloid polypeptide (IAPP), calcitonin, a prion, atrial natriuretic factor (ANF), lysozyme, insulin, fibrinogen, or α-synuclein.

Incubation conditions may be determined by the person skilled in the art according to conventional procedures. The separation of the two components of the fusion protein also includes that either or both components are degraded to a certain extent. It is, however, important that the capability of the (poly)peptide that has the ability of self-assembling is not lost due to the degradation.

Preferably, the cleavable site is an enzymatically cleavable site or a chemically cleavable site or a site cleavable by intein self-cleavage in the presence of thiols. A number of enzymes that site-specifically or non-site-specifically digest proteinaceous material is known in the art. Examples of such enzymes are factor Xa, thrombin, trypsin, endopeptidases Arg C or Lys C, proteinase K or elastase. Depending on the goal of the experiment, any or most of the known enzymes are applicable to this test. The same holds true for most of the chemical cleavage agents.

In accordance with the present invention, it is most preferred that the method of the invention further comprises, prior to step (b) and after step (a"):
(a"') incubation with an inhibitor of said compound that induces cleavage.
This embodiment of the method of the invention allows precise incubation periods of the cleaving agent which may be useful, for example, for studying the kinetics of aggregation.
A general overview of the above-recited specific embodiments of the invention relating to the cleaving of fusion protein are detailed in Fig. 7.

It is furthermore particularly preferred that said amyloidogenic (poly)peptide comprises a polyglutamine expansion. As has been demonstrated in the appended examples, the specific embodiments relating to the cleavage of fusion proteins may be advantageously employed for determining fibril or aggregate formation of proteins that comprise polyglutamine expansions. Accordingly, this embodiment is particularly useful for the assessment of the onset or the progress of Huntington's disease, spinal and balba muscular atrophy, dentatorubral pallidoluysian atrophy and the spinocereberal ataxia types 1, 2, 3 and 6.

Further particularly preferred is that the polyglutamine expansion comprises at least 35, preferably at least 41, more preferably at least 48 and most preferably at least 51 glutamines.

The length of the polyglutamine expansions appears to be correlated to the susceptibility of humans or animals to the above-recited diseases. In this regard, we also refer to the European patent application entitled "Novel composition and method for the detection of diseases associated with amyloid-like fibril or protein aggregate formation" filed on the same day with the European Patent Office and assigned to the same applicant. The contents of said application are specifically incorporated herein by reference.

Further preferred is in accordance with the present invention that said contacting is effected by dotting, spotting or pipetting said material onto said filter.
This embodiment is particularly useful for an automated application of the invention. Additionally, the set-up associated with dot-blotting or spot-blotting allows the investigation of a large number of sample materials as well as the cost-conscious application of the method of the invention. This is due to the fact that a large number of samples can be assessed for fibril or aggregate formation on one and the same filter.

It is furthermore preferred that the filter is a filter membrane which is optionally or preferably contained in a microtitre plate. Additionally preferred is the use of SDS as detergent or Triton X-100 for non-β-amyloid aggregates.

The invention further relates to an inhibitor identified by the method of the invention. While practically any compound class may be tested for inhibitory effects, it is preferred that said inhibitor is an antibody or a derivative or functional fragment thereof, a peptide or a chemical reagent.

Furthermore, the present invention relates to a pharmaceutical composition comprising the inhibitor of the invention and pharmaceutically acceptable carrier and/or diluent. Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Compositions comprising such carriers can be formulated by well known conventional methods. These pharmaceutical compositions can be administered to the subject at a suitable dose. Administration of the suitable compositions may be effected by different ways, e.g., by intravenous, intraperitoneal, subcutaneous,

intramuscular, topical or intradermal administration. The dosage regimen will be determined by the attending physician and other clinical factors. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. A typical dose can be, for example, in the range of 0.001 to 1000 µg (or of nucleic acid for expression or for inhibition of expression in this range); however, doses below or above this exemplary range are envisioned, especially considering the aforementioned factors. Generally, the regimen as a regular administration of the pharmaceutical composition should be in the range of 1 µg to 10 mg units per day. If the regimen is a continuous infusion, it should also be in the range of 1 µg to 10 mg units per kilogram of body weight per minute, respectively. Progress can be monitored by periodic assessment. Dosages will vary but a preferred dosage for intravenous administration of DNA is from approximately 10^6 to 10^{12} copies of the DNA molecule. The compositions of the invention may be administered locally or systemically. Administration will generally be parenterally, e.g., intravenously; DNA may also be administered directly to the target site, e.g., by biolistic delivery to an internal or external target site or by catheter to a site in an artery.

The therapeutically useful compounds identified according to the method of the invention may be administered to a patient by any appropriate method for the particular compound, e.g., orally, intravenously, parenterally, transdermally, transmucosally, or by surgery or implantation (e.g., with the compound being in the form of a solid or semi-solid biologically compatible and resorbable matrix) at or near the site where the effect of the compound is desired.

The invention further relates to diagnostic composition comprising
(i) a fusion protein as defined in the invention.

Preferably, the diagnostic composition further comprises
(ii) a filter as defined in the invention optionally or preferably combined in a microtitre plate; and optionally

(iii) a compound that induces cleavage as defined in any one of the preceding claims; and optionally
(iv) an inhibitor of said compound of (c); and optionally
(v) suitable buffer solutions.

The diagnostic composition of the invention may be used for a variety of purposes. For example, it may be used for detecting the presence, etiology or status of one of the above-mentioned diseases or a corresponding disease state in a patient. In addition, it may be used for the development of suitable inhibitors of the formation of amyloid-like fibrils or protein aggregates that are preferably, but not exclusively, associated with the above-recited disease states.

The components of the composition of the invention may be packaged in containers such as vials, optionally in buffers and/or solutions. If appropriate, one or more of said components may be packaged in one and the same container.

The figures show:

Figur 1
SDS-PAGE Analysis of Purified GST and GST-HD Fusion Proteins.

(a) Aliquots (15 ml) of eluates from the glutathione agarose column were subjected to 12.5 % SDS-PAGE and analyzed by staining with Coomassie blue R. Lanes 1-6 contain GST, GST-HD20, -HD30, -HD83 and -HD122, respectively; lane M contains molecular mass standards. (b) Proteins were transferred to nitrocellulose and probed with anti-HD1 antibody. Arrows mark the origin of electrophoresis.

Figure 2
Structure of GST-HD fusion proteins.

The amino acid sequence corresponding to exon 1 of huntingtin is boxed. Arrows labeled Xa and T indicate cleavage sites for factor Xa and trypsin, respectively.

Figure 3
Site-Specific Proteolysis of GST-HD Fusion Proteins with Trypsin and Factor Xa.

Tryptic digestions were performed at 37°C for 3 (a) or 16 h (b). Native proteins and their cleavage products were subjected to 12.5% SDS-PAGE, blotted onto nitrocellulose membranes, and probed with anti-HD1 antibody. Arrows mark the origin of electrophoresis. (c) Purified fusion proteins and their factor Xa and trypsin cleavage products were analyzed using the filter retardation assay. The proteins retained by the cellulose acetate and nitrocellulose membranes were detected by incubation with the anti-HD1 antibody.

Figure 4
Electron Micrographs of Native GST-HD Fusion Proteins and their Factor Xa and Trypsin Cleavage Products.

Purified GST fusion proteins were protease treated, negatively stained with uranyl acetate and viewed by electron microscopy. The undigested GST-HD51 molecules appear as a homogeneous population of small, round particles (a).

Removal of the GST-tag with factor Xa results in the formation of amyloid-like fibrils and intermediate structures (b + c). After partial digestion (3 h) of GST-HD51 with trypsin, the ribbons are associated with terminal clots (d, arrow), whereas prolonged digestion (16 h) produces ribbons without attached clots (e). Removal of the GST-tag from GST-HD20 shows no evidence for the formation of defined structures (f).

Figure 5
Birefringence of Protein Aggregates Formed by Proteolytic Cleavage of GST-HD51.

The protein aggregates were stained with Congo red. (a) Bright field, 200x; (b) Polarized light, 200x; (c) Polarized light, 100x.

Figure 6
Polygln-Containing Protein Aggregates are Formed *in vivo*.

(a) Western blot analysis, after separation by 10% SDS-PAGE, of the nuclear (N) and cytosolic (C) protein fractions prepared from brain and kidney of an R6/2 hemizygous transgenic mouse and a littermate control. Blots were probed with anti-HD1, anti-GAPDH and anti-Fos B antibodies as indicated. (b) Detection of HD exon 1 protein aggregates formed *in vivo* using the cellulose acetate filter assay. The membrane was immunostained using the anti-HD1 antibody. (c) Ultrastructure of a neuronal intranuclear inclusion (NII). The presence of a NII in a striatal neuron of a 17 month old R6/5 homozygous mouse is shown. The NII is indicated by the large arrow and the fibrillar amyloid-like structures within the NII are indicated by two small arrows. The scale bar is 250 nm.

Figure 7

A: Purification of GST-HD fusion proteins containing polyglutamine expansions by affinity chromatography
B: Transfer of soluble GST-HD fusion protein into a microtiter plate using a pipetting robot
C: Transfer of various inhibitors into the microtiter plate using a pipetting robot

D: Transfer of a protease to the microtiter plate using a pipetting robot to start the formation of insoluble protein fibrils. Incubation of the microtiter plate at 25°C-37°C to allow fibril formation

E: Addition of a protease inhibitor to stop the cleavage reaction using a pipetting robot

F: Transfer of the reaction mixtures onto a cellulose acetate (CA) and a nitrocellulose membrane (NC) using a spotting robot or a pipetting robot

G: Washing of the CA membrane with SDS-buffer to remove soluble proteins and the NC membrane with blocking buffer

H: Detection of the proteins bound to the CA and NC membranes by Western blot analysis using a specific antibody

I: Comparison of the membranes and identification of compounds that block fibril formation using specific computer programs

Figure 8

Structure of GST-HD fusion proteins. The amino acids sequence corresponding to the N-terminal portion of huntingtin is boxed and the amino acids corresponding to the biotinylation site are underlined. Arrows labeled (Xa) and (T) indicate cleavage sites for factor Xa and trypsin, respectively.

Figure 9

Detection of polyglutamine-containing protein aggregates formed *in vitro* and in transfected COS-1 cells using the dot-blot filter retardation assay. (A) Purified GST-HD20DP and -HD51DP fusion proteins (250 ng) and their factor Xa and trypsin cleavage products were applied to the filter as indicated. The aggregated proteins retained by the cellulose acetate membrane were detected by incubation with the anti-HD1 antibody. (B) Scanning electron micrograph of aggregated GST-HD51DP trypsin cleavage products retained on the surface of the cellulose acetate membrane (Photo: Heinrich Lündsdorf, GBF Braunschweig, Germany). (C) Dot-blot filter retardation assay performed on the insoluble fraction isolated from transfected and non-transfected COS-1 cells. COS-1 cells were transiently transfected with the plasmids pTL1-CAG20, -CAG51 and CAG93 encoding huntingtin exon 1 proteins with

20 (HD20), 51 (HD51) and 93 (HD93) glutamines, respectively. The pellet fractions obtained after centrifugation of whole cell lysates were subjected to DNaseI/trypsin digestion, boiled in 2% SDS, and portions of 1, 3 and 6 µl were filtered through a cellulose acetate membrane. The aggregated huntingtin protein retained on the membrane was detected with the anti-HD1 antibody. NT, non-transfected cells.

Figure 10

Detection and quantification of aggregates formed *in vitro* from biotinylated GST-HD exon 1 fusion proteins. Various amounts of the fusion proteins GST-HD51DPBio and -HD20DPBio were filtered through a cellulose acetate membrane after a 3-h incubation at 37°C in the presence or absence of trypsin as indicated. (A) Images of the retained protein aggregates, detected with streptavidin-AP conjugate using either a fluorescent (upper panel) or a chemiluminescent AP substrate (lower panel). (B) Quantification of signal intensities obtained for the GST-HD51DPBio dots seen in *A*.. Fluorescence and chemiluminescence values are arbitrary units generated by the Lumi-Imager F1 and LumiAnalyst™ software (Boehringer Mannheim).

Figure 11

Detection (A) and quantification (B) of aggregates formed *in vitro* from biotinylated GST-HD exon 1 fusion proteins using the dot-blot and microtitre plate filter retardation assay. Various amounts of the fusion proteins GST-HD51DPBio and -HD20DPBio were filtered through the cellulose acetate membranes after a 3-h incubation at 37°C in the presence or absence of trypsin as indicated. The detection and quantification of the aggregates was as described in Fig. 3.

Figure 12

Detection of neurofibrillar tangles (NFTs) and β-amyloids in brain extracts prepared from Alzheimer's disease patients and controls using the dot-blot filter retardation assay. The cellulose acetate membrane was probed with the polyclonal anti-Tau, the

monoclonal anti-β-amyloid, or the polyclonal anti-HD antibody. A1, A2, and A3: protein extracts prepared from cerebral cortex of Alzheimer's disease patients; C1, C2, and C3: protein extracts prepared from cerebral cortex of normal individuals. GST-HD51, fusion of glutathione S-transferase and huntingtin exon 1 containing 51 glutamines.

The examples illustrate the invention:

Example 1:
Purification of GST-HD fusion proteins containing expanded polygIns

Exon 1 of the HD gene was isolated from genomic phage clones, derived from the normal and expanded alleles of an HD patient (Sathasivam et al., 1997), and used for the expression of GST-HD fusion proteins in *E. coli*. DNA fragments containing CAG repeats in the normal $(CAG)_{20-33}$ and expanded $(CAG)_{37-130}$ range were cloned into pGEX-5X-1 (Pharmacia), and the resulting plasmids expressing fusion proteins with 20 (GST-HD20), 30 (-HD30), 51 (-HD51), 83 (-HD83) and 122 (-HD122) glutamines, respectively, were used for protein purification. For plasmid construction lambda phage from stock 9197_4 (Sathasivam et al., 1997) were plated to give single plaques which were innoculated into 400 ml cultures of *E. coli* XL1-Blue MRF' (Stratagene) for DNA preparation. The DNA sequence encoding the N-terminal portion of huntingtin (exon 1), including the CAG repeats, was amplified by PCR using the following pair of primers: ES 25 (TGGGATCCGCATGGCGACCCTGGAAAAGCTGATGAAGG) corresponding to nt315-343 of the HD gene (HDCRG, 1993) and containing a BamHI site (underlined) and ES 26 (GGAGTCGACTCACGGTCGGTGCAGCGGCTCCTCAGC) corresponding to nt516-588 and containing a SalI site (underlined). Conditions for PCR were as described (Mangiarini et al. 1996). Due to instabiltity of the CAG repeat during propagation in *E. coli* , DNA preparations from individual plaques yielded different sized PCR products. Fragments of ~ 320, 360, 480, and 590 bp were gel-purified, digested with BamHI and SalI and inserted into the BamHI-SalI site of the expression vector pGEX-5X-1 (Pharmacia), yielding pCAG30, pCAG51, pCAG83 and pCAG122, respectively. pCAG20, containing 20 repeats of CAG within the cloned HD exon 1 sequence, was similarly constructed from a phage genomic clone derived from a normal allele. All constructs were verified by sequencing. After induction with IPTG, the resulting proteins were purified under native conditions by affinity chromatography on glutathione agarose. Thus, *E. coli* SCS1 (Stratagene) carrying the pGEX expression plasmid of interest was grown to an OD_{600nm} of 0.6 and induced with IPTG (1 mM) for 3.5 h as described in the manufacturer's protocol (Pharmacia). Cultures (200 ml) of induced bacteria were centrifuged at 4000 g for 20 min, and the resulting pellets were stored at -

80°C. Cells were thawed on ice and resuspended in 5 ml of lysis buffer (50 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, pH 7.4) containing 0.5 mg/ml lysozyme. After 45 min at 0°C, cells were sonicated with two 30 sec-bursts. Octyl-β-D-glucopyranoside was then added to a final concentration of 0.1% and the resulting lysate was clarified by centrifugation at 30,000 g for 30 min at 4°C. Cleared lysates were incubated for 1 h at 4°C with 500 µl of a 1:1 slurry of glutathione-agarose beads (Sigma) that had been washed times and resuspended in lysis buffer. The beads were poured into a small column and washed extensively with lysis buffer containing 0.1% octyl-β-D-glucopyranoside. The bound fusion protein was eluted with 2 ml of 15 mM glutathione (reduced) in lysis buffer. Typical yields were 0.5-1 mg of purified GST-HD20, -HD30 and -HD51 proteins per 200 ml of bacterial culture; yields of GST-HD83 and -HD122 were much lower, less than 10% of that obtained with the shorter fusion proteins. Protein was determined by the Bio-Rad dye binding assay using bovine serum albumin as standard. SDS-PAGE of the purified GST-HD20, -HD30, -HD51, -HD83 and -HD122 proteins revealed major bands of 42, 45, 50, 65 and 75 kDa, respectively (Fig. 1a). These bands were also detected when the various protein fractions were subjected to immunoblot analysis using the affinity purified anti-huntingtin antibody HD1 (Fig. 1b, lanes 2-6). HD1 specifically detects the GST-HD fusion proteins on immunoblots, whereas the GST-tag alone is not recognized (Fig. 1b, lane 1). For immunoblotting a bacterial plasmid encoding HD1-His, a His_6-tagged fusion protein containing residues 1-222 of huntingtin, was generated by inserting a PCR-amplified *IT-15* cDNA fragment into the pQE-32 vector (Qiagen). The fusion protein was expressed in *E. coli*, affinity-purified under denaturating conditions on Ni-NTA agarose, and injected into rabbits. The resulting immune serum was then affinity-purified against the antigen that had been immobilized on Ni-NTA agarose. The GAPDH- and Fos B-specific antisera have been described (Wanker et al., 1997; Davies et al., 1997).

Western blotting was performed as detailed (Towbin et al., 1979). The blots were incubated with 1:1000 dilutions of the indicated primary antibody, followed by an alkaline-phosphatase-conjugated secondary antibody. Color development was carried out with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as substrates (Promega).

All recombinant proteins migrated at a size corresponding nearly to that predicted from their amino acid sequence. Interestingly, an additional high molecular weight

band which remains at the top of the gel, was consistently detected in the protein fractions with the longest polyglns (83 and 122 residues; Fig. 1a and b, lane 5 and 6). This band was most prominent on the immunoblots but was also clearly detectable in the Commassie stained gel. This immunoreactive material was often still present at the bottom of the loading slots, even after the samples had been boiled for 5 min in the presence of 2% SDS and 6 M urea prior to loading.

Example 2:
Proteolytic cleavage of GST-HD fusion proteins containing expanded polyglns

It has been shown previously that the solubility of certain proteins can be enhanced by the addition of the GST-tag (Smith and Johnson, 1988) and it was therefore of interest to determine whether the removal of the GST-tag by proteolytic cleavage would have an effect on the solubility of the polygln-containing fusion proteins. Potential factor Xa and trypsin cleavage sites within the GST-HD fusion proteins are shown in Fig. 2. Factor Xa cleaves between the GST-tag and the HD exon 1 protein whereas trypsin removes an additional 15 amino acids from the N-terminus and a single proline from the C-terminus, both proteases leaving the polygln repeat intact. The GST-HD20, -HD30 and -HD51 proteins were digested with trypsin under conditions designed to remove the GST-tag from the fusion protein without it being totally degraded. After cleavage, proteins were denatured by boiling in the presence of 2% SDS and analyzed by SDS-PAGE and immunoblotting using the anti-HD1 antibody. GST-HD20 and -HD30 cleavage yielded products migrating in a 12.5 % gel at approximately 30 and 33 kDa, respectively. In contrast, cleavage of GST-HD51 resulted in the formation of two protein products migrating at approximately 37 and 60 kDa, and an additional weak immunoreactive band on the bottom of the loading slots was also detected (Fig. 3a). This high molecular weight band was more pronounced when GST-HD51 was digested with trypsin under conditions in which the GST-tag was totally degraded (Fig. 3b). However, with proteins GST-HD20 and -HD30 this longer exposure to trypsin produced the same cleavage products as the ones seen in Fig. 3a and the high molecular weight products were not observed. Similar results were obtained with factor Xa protease and endoproteinases Arg-C and Lys-C. As regards the proteolytic cleavages, the following protocols were carried out: The GST-HD fusion proteins purified as described above were dialysed against 40 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1 mM EDTA and 5%

(v/v) glycerol to raise the pH prior to proteolytic cleavage. The proteins were then combined with bovine factor Xa (New England Biolabs) or modified trypsin (Boehringer Mannheim, sequencing grade) in dialysis buffer containing 2 mM $CaCl_2$ at an enzyme:substrate ratio of 1:10 (w/w) or 1:40 (w/w), respectively. Incubations with factor Xa were at 25°C for 16 h. Tryptic digestions were performed at 37°C for 3 or 16 h as indicated. Digestions were terminated by the addition of PMSF to 1 mM . The degree of proteolysis was determined by SDS-PAGE followed by staining with Coomassie blue or immunoblottting using anti-HD1 antibody.

We have developed a simple and sensitive filter assay to detect the formation of high molecular weight insoluble protein aggregates. This assay is based on the finding that the SDS-insoluble protein aggregates obtained by proteolytic cleavage of GST-HD51 are retained on a cellulose acetate filter, whereas the soluble cleavage products of GST-HD20 and GST-HD30 are not. Factor Xa or trypsin digestions of purified GST-HD fusion proteins (10 µg) were performed in a 20 µl reaction mixture as described above. Reactions were terminated by adjusting the mixture to 2% SDS and 50 mM DTT. After heating at 100°C for 5 min, aliqouts (0.5 µl) were diluted into 200 µl of 0.1% SDS and filtered through a cellulose acetate membrane (Schleicher & Schuell, 0.2 µm pore size) using a BRL dot blot filtration unit. Filters were washed with water, and the SDS-insoluble aggregates retained on the filter detected by incubation with the anti-HD1 antibody, followed by an anti-rabbit secondary antibody conjugated to alkaline phosphatase (Boehringer Mannheim). Fig. 3c shows immunoblots of cellulose acetate and nitrocellulose membranes to which the native GST-HD20, -HD30 and -HD51 proteins and their factor Xa and trypsin cleavage products have been applied. On the cellulose acetate filter, only the cleavage products of GST-HD51 were detected by the anti-HD1 antibody, indicating the formation of insoluble high molecular weight protein aggregates. In contrast, all the uncleaved GST-HD fusion proteins and their digestion products were detected on the nitrocellulose control filter. This assay was also used to detect huntingtin aggregates present in a nuclear fraction from the brain of an R6/2 hemizygous mouse and littermate control (see preparation of nuclei below).

Exampl 3:

Huntingtin proteins containing expanded polyglns in the pathological range aggregate to amyloid-like birefringent fibrils

Electron microscopy of negatively stained GST-HD51 fractions showed oligomeric particles with diameters of 6 to 7 nm (Fig. 4a); no higher ordered aggregates were observed. For electron microscopic observation, the native or protease-digested GST-HD fusion proteins were adjusted to a final concentration of 50 µg/ml in 40 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1 mM EDTA and 5% glycerol. Samples were negatively stained with 1% uranyl acetate and viewed in a Philips CM100 EM. In contrast, protein fractions obtained by proteolytic cleavage of GST-HD51 showed numerous clusters of high molecular weight fibrils and ribbon-like structures (Fig. 4b, c, d and e), reminiscent of purified amyloids (Prusiner et al., 1983). The fibrils obtained after digestion with factor Xa showed a diameter of 10-12 nm and their length varied from 100 nm up to several micrometers (Fig. 4b and c). In the trypsin-treated samples ribbon-like structures formed by lateral aggregation of fibrils with a diameter of 7.7 nm were observed (Fig. 4d and e). After treatment with factor Xa or limited digestion with trypsin, clots of small particles were frequently detected on one or both ends of the fibrils (Fig. 4b, c and d). These clots of varying sizes and shapes were not seen when GST-HD51 was digested with trypsin under conditions in which the GST-tag is totally degraded (Fig. 4e), indicating that they contain GST. In strong contrast to GST-HD51, the GST-HD20 and -HD30 proteins did not show any tendency to form ordered high molecular weight structures, either with or without protease treatment (Fig. 4f).

The insoluble protein aggregates formed by proteolytic cleavage of GST-HD51 were isolated by centrifugation and stained with Congo red (Caputo et al., 1992) and examined under a light microscope. For light microscopy, peptide aggregates formed by trypsin digestion of purified GST-HD fusion proteins (50 µg in 100 µl of digestion buffer) were collected by centrifugation at 30,000 g for 1 h and resuspended in 10 µl of water. Samples were mixed with 0.1 volume of a 2% (w/v) aqueous Congo Red (Sigma) solution, placed on aminoalkylsilane-coated glass slides, and allowed to dry overnight under a coverslip. After removing the coverslip, excess Congo Red was removed by washing with 90% ethanol. Evaluation of the Congo Red staining by polarization microscopy was performed using a Zeiss Axiolab Pol microscope equipped with strain-free lenses and optimally aligned cross-polarizers. After staining, the protein aggregates on the

glass slides were red, indicating that they had bound the dye (Fig. 5a), and when examined under polarized light a green color and birefringence were detected (Fig. 5b and c). These staining characteristics were similar to those observed for prions (Prusiner et al., 1983) and amyloids (Caputo et al., 1992).

Example 4:
Huntingtin proteins containing expanded polyglns form amyloid-like protein aggregates *in vivo*

To determine whether the amyloid-like protein aggregates formed by proteolytic cleavage of GST-HD51 *in vitro* are also present *in vivo*, nuclear protein fractions of brain and kidney were prepared from mice transgenic for the HD mutation (line R6/2) and littermate controls (Davies et al., 1997; Mangiarini et al., 1996). Nuclei from the brain or kidney of an R6/2 hemizygous mouse with a repeat expansion of $(CAG)_{143}$ (Mangiarini et al., 1996) at ten weeks of age and littermate control were prepared as follows. Whole brain samples (80 mg) in 400 ml of 0.25 M sucrose in buffer A (50 mM triethanolamine [pH 7.5], 25 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF) were homogenized using 15 strokes of a tight-fitting glass homogenizer. The homogenate was adjusted to a final concentration of 5 mM DTT, and centrifuged at 800 g for 15 min. The supernatant was recentrifuged at 100,000 g for 1 h, and the supernatant from this centrifugation was taken as the cytosolic fraction (fraction C). The loose pellet from the first centrifugation was homogenized, diluted to 1.2 ml with 0.25 M sucrose/buffer A, and mixed with two volumes of 2.3 M sucrose/buffer A. The mixture was then layered on top of 0.6 ml 2.3 M sucrose/bufferA in a SW60 tube and centrifuged at 124,000 g for 1 h. The pellet was harvested with a spatula, resuspended in 200 µl of 0.25 M sucrose/buffer A and again centrifuged at 800 g for 15 min. The entire procedure was carried out at 4 °C. The pelleted nuclei were resuspended to a density of ~ 1 x 10^7 nuclei/ml in 0.25 sucrose/buffer A (fraction N) and stored at -80 °C. Nuclei from mouse kidney were prepared in the same way. The protein extracts were analyzed by SDS-PAGE and Western blotting using the anti-HD1 antibody (Fig. 6a). Strikingly, this antibody detected a prominent high molecular weight band in the nuclear fraction (N) prepared from R6/2 transgenic brain, very similar to the high molecular weight band obtained by proteolytic cleavage of GST-HD51 (Fig. 3b). No such immunoreactive band was detected in the nuclear fraction of brain from the littermate control and it was also absent from the corresponding cytoplasmic fractions (C). A small amount of high molecular weight material was

also detected in the nuclear fraction prepared from R6/2 transgenic kidney, but was again absent from the cytoplasmic fraction. The purity of the nuclear and cytoplasmic fractions was confirmed by Western blot analysis using the anti-Fos B and anti-GAPDH antibodies. Anti-Fos B detected the transcription factor mainly in the nuclear fraction, and the enzyme GAPDH was only seen in the cytoplasmic fraction, as expected. The Western blot results were reproduced using the cellulose acetate filter assay (Fig. 6b). Using this assay, a 10-20 fold higher amount of transgene protein was detected in the nuclear fraction isolated from brain material, compared to that prepared from kidney.

The formation of NIIs has been shown to preceed the neuronal dysfunction that forms the basis of the progressive neurological phenotype observed in the R6 transgenic lines (Davies et al., 1997). These NIIs are immunoreactive for both huntingtin and ubiquitin antibodies and contain the transgene but not the endogenous huntingtin protein. Therefore, Western blot analysis using an anti-ubiquitin antibody was also performed showing the same pattern of immunoreactivity as had been observed with the anti-HD1 antibody (Fig. 6a), and indicating that the high molecular weight transgene protein present in the nuclear fraction is ubiquitinated (data not shown).

To examine whether the NIIs containing the proteins huntingtin and ubiquitin (Davies et al., 1997) have a fibrous composition, an ultrastructural analysis was performed. Experimentally, a 17 month old R6/5 homozygous mouse ($(CAG)_{128-155}$) (Mangiarini et al., 1996) was deeply anaesthetised with sodium pentobarbitone and then perfused through the left cardiac ventricle with 35-50 ml of 4 % paraformaldehyde and either 0.5 % glutaraldehyde in 0.1 M Millonig's phosphate buffer (pH 7.4). The brain was removed from the skull and placed in fresh fixative overnight at 4 °C. Coronal sections (50 - 200 µm) were cut on an Oxford Vibratome (Lancer) and collected in serial order in 0.1 M phosphate buffer. After being osmicated (30 min in 1% OsO_4 in 0.1 M phosphate buffer) the sections were stained for 15 min in 0.1 % uranyl acetate in sodium acetate buffer at 4 °C, dehydrated in ethanols, cleared in propylene oxide and embedded in Araldite between two sheets of Melanex (ICI). Semi thin (1 µm) sections were cut with glass knives and stained with toluidine blue adjacent to thin sections cut with a diamond knife on a Reichert Ultracut ultramicrotome. The sections were collected on mesh grids coated with a thin formvar film, counterstained with lead citrate and viewed in a Jeol 1010 electron microscope. An electron micrograph of

a NII from a 17 month old R6/5 homozygous mouse is shown in Fig. 6c. This NII (large arrow) contains high molecular weight fibrous structures which were clearly differentiated from the surrounding chromatin. The filaments were randomly oriented, 5-10 nm in diameter and often measured up to 250 nm in length (small arrows). These structures differ from those previously reported in the NIIs seen in hemizygous R6/2 mice which were far more granular in composition, with individual filamentous structures being more difficult to distinguish (Davies et al., 1997). R6/2 mice exhibit an earlier age of onset with a more rapid progression of the phenotype and do not survive beyond 13 weeks (Mangiarini et al., 1996). It is possible that the filamentous structures do not have time to form in the R6/2 mice.

Example 5:

Construction of further plasmids, purification of corresponding GST fusion proteins and proleolytic cleavage of GST fusion proteins

In a second set of experiments, a further number of plasmids was constructed. Standard protocols for DNA manipulations were followed (J. Sambrook, E.F. Fritsch, and T. Maniatis, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Plainview, NY, 1989). *IT-15* cDNA sequences (HDCRG, *Cell* **72**, 971 (1993)) encoding the N-terminal portion of huntingtin, including the CAG repeats, were amplified by PCR using the oligonucleotides ES25 (5'-TGGGATCCGCATGGCG
ACCCTGGAAAAGCTGATGAAGG-3') and ES27 (3'-CTCCTCGAGCGGCGG
TGGCGGCTGTTGCTGCTGCTGCTG-5') as primers and the plasmids pCAG20 and pCAG51 as template (E. Scherzinger, R. Lurz, M. Trumaine, L. Margiarini, B. Hollenbach, R. Hasenbank, G. P. Bates, S. W. Davies, H. Lehrach, and E. E. Wanker, *Cell* **90**, 549 (1997)). Conditions for PCR were as described (L. Mangiarini, K. Sathasivam, M. Seller, B. Cozens, A. Harper, C. Hetherington, M. Lawton, Y. Trottier, H. Lehrach, S.W. Davies, and G.P. Bates, *Cell* **87**, 493 (1996)). The resulting cDNA fragments were gel purified, digested with *Bam* HI and *Xho* I and were inserted into the *Bam* HI-*Xho* I site of the expression vector pGEX-5X-1 (Pharmacia), yielding pCAG20DP and pCAG51DP, respectively. The plasmids pCAG20DP-Bio and pCAG51DP-Bio were generated by subcloning the PCR fragments obtained from the

plasmids pCAG20 and pCAG51 into pGEX-5X-1-Bio. pGEX-5X-1-Bio was created by ligation of the oligonucleotides BIO1 (5'-CGCTCGAGGGTATCTTCGAGGCCC AGAAGATCGAGTGGCGATCACCATGAG-3') and BIO2 (5'-GGCCGCTCATGGTG ATCGCCACTCGATCTTCTGGGCCTCGAAGATACCCTCGAG-3'), after annealing and digestion with *Xho* I, into the *Xho* I-*Not* I site of pGEX-5X-1. The plasmids with the *IT-15* cDNA inserts were sequenced to confirm that no errors had been introduced by PCR. The construction of plasmids pTL1-CAG20, pTL1-CAG51 and pTL1-CAG93 for the expression of huntingtin exon 1 proteins containing 20, 51 and 93 glutamines in mammalian cells has been described (A. Sittler, S. Wälter, N. Wedemeyer, R. Hasenbank, E. Scherzinger, G. P. Bates, H. Lehrach, and E. E. Wanker, *Mol. Cell* , submitted).

The amino acid sequence of the GST-HD fusion proteins encoded by the *E. coli* expression plasmids pCAG20DP, pCAG51DP, pCAG20DP-Bio and pCAG51DP-Bio is shown in Fig. 8. The plasmids pCAG20DP and pCAG51DP encode fusion proteins of glutathione S-transferase (GST) and the N-terminal portion of huntingtin containing 20 (GST-HD20DP) and 51 (-HD51DP) polyglutamines, respectively. In these proteins the proline-rich region located immediately downstream of the glutamine repeat was deleted (E. Scherzinger, R. Lurz, M. Trumaine, L. Margiarini, B. Hollenbach, R. Hasenbank, G. P. Bates, S. W. Davies, H. Lehrach, and E. E. Wanker, *Cell* **90**, 549 (1997)). The fusion proteins GST-HD20DPBio and -HD51DPBio are identical to GST-HD20DP and -HD51DP, except for the presence of a biotinylation site (P. J. Schatz, *Biotechnology* **11**, 1138 (1993)) at their C-termini.

In the experiments described herein, *E. coli* DH10B (BRL) was used for plasmid construction and *E. coli* SCS1 (Stratagene) was used for the expression of GST-HD fusion proteins. Transformation of *E. coli* with plasmids and ligation mixtures was performed by electroporation using a Bio-Rad Gene Pulser (Richmond, CA). Transformed cells were spread on LB plates supplemented with appropriate antibiotics (J. Sambrook, E.F. Fritsch, and T. Maniatis, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Plainview, NY, 1989). For expression of GST fusion proteins, cells were grown in liquid TY medium

(5 g NaCl, 5 g yeast extract, and 10 g tryptone per liter) buffered with 20 m*M* MOPS/KOH (pH 7.9) and supplemented with glucose (0.2%), thiamine (20 μg/ml), ampicillin (100 μg/ml) and kanamycin (25 μg/ml).

The procedure for purification of GST fusion proteins is an adaption of the protocol of Smith and Johnson (D. B. Smith and K. S. Johnson, *Gene* **67**, 31 (1988)). Unless indicated otherwise, all steps were performed at 0-4°C.

First, 100 ml TY medium were inoculated with a single colony containing the expression plasmid of interest, and the culture was incubated at 37°C overnight with shaking. Then, 1.5 liter TY medium were inoculated with the overnight culture and grown at 37°C until an OD_{600} of 0.6 was reached. IPTG was added to a final concentration of 1 m*M*, and the culture continued to grow at 37°C for 3.5 h with vigorous shaking. The culture was chilled on ice, and the cells harvested by centrifugation at 4000 x g for 20 min.

Cells were washed with buffer A [50 m*M* sodium phosphate (pH 8), 150 m*M* NaCl, and 1 m*M* EDTA]. If neccessary, the cell pellet was stored at -70°C. Cells were resuspended in 25 ml buffer A. PMSF and lysozyme (Boehringer Mannheim) were added to 1 m*M* and 0.5 mg/ml, respectively, and incubated on ice for 45 min. Cells were lysed by sonication (2 x 45 s, 1 min cooling, 200-300 Watt), and Triton X-100 was added to a final concentration of 0.1% (v/v). The lysate was centrifuged at 30.000 x g for 30 min, and the supernatant was collected.

5 ml of a 1:1 slurry of GST-agarose (Sigma), previously equilibrated in buffer A, was added and the mixture was stirred for 30 min. The slurry was poured into a 1.6 cm diameter column, washed once with 40 ml buffer A containing 1 m*M* PMSF and 0.1 % Triton X-100 and twice with 40 ml buffer A containing 1 m*M* PMSF. The protein was eluted with 5 x 2 ml buffer A containing 15 m*M* reduced glutathione (Sigma). Aliquots of the fractions were analyzed by SDS-PAGE and the fractions containing purified GST fusion protein were combined. Finally, the pooled fractions were dialysed

overnight against buffer B [20 m*M* Tris/HCl (pH 8), 150 m*M* NaCl, 0.1 m*M* EDTA and 5 % (v/v) glycerol], aliquotted, freezed in liquid nitrogen and stored at -70°C.

Typical yields were 10 - 20 mg for GST-HD20DP and -HD51DP and 5-10 mg for GST-HD20DPBio and -HD51DPBio per liter of bacterial culture. Protein concentration was determined using the Coomassie protein assay reagent from Pierce with BSA as a standard.

The GST-huntingtin fusion proteins (2 mg) were digested with bovine factor Xa (New England Biolabs) or with modified trypsin (Boehringer Mannheim, sequencing grade) at an enzyme/substrate ratio of 1:10 (w/w) and 1:20 (w/w), respectively. The reaction was carried out in 20 µl of 20 m*M* Tris/HCl (pH 8), 150 m*M* NaCl and 2 m*M* $CaCl_2$. Incubations with factor Xa were performed at 25°C for 16 h. Tryptic digestions were at 37°C for 3 to 16 h. Digestions were terminated by the addition of 20 µl 4% (w/v) SDS and 100 m*M* DTT, followed by heating at 98°C for 5 min.

As shown in the previous examples, removal of the GST tag from the HD exon 1 protein containing 51 glutamines (GST-HD51) by site-specific proteolytic cleavage results in the formation of high molecular weight protein aggregates, seen as characteristic fibrils or filaments on electron microscopic examination. Such ordered fibrillar structures were not detected after proteolysis of fusion proteins containing only 20 (GST-HD20) or 30 (GST-HD30) glutamines, although light scattering measurements (Y. Georgalis, E.B. Starikov, B. Hollenbach, R. Lurz, E. Scherzinger, W. Saenger, H. Lehrach, and E.E. Wanker, *Proc. Natl. Acad. Sci. USA* **95**, 6118 (1998)) revealed that some form of aggregation also occured with these normal repeat-length proteins. In the present example, truncated GST-HD exon 1 fusion proteins with or without a C-terminal biotinylation tag (P. J. Schatz, *Biotechnology* **11**, 1138 (1993) were used. These fusion proteins contain either 20 or 51 glutamines but lack most of the proline rich region located downstream of the glutamine repeat (E. Scherzinger, R. Lurz, M. Trumaine, L. Margiarini, B. Hollenbach, R. Hasenbank, G. P. Bates, S. W. Davies, H. Lehrach, and E. E. Wanker, *Cell* **90**, 549 (1997)). Potential factor Xa and trypsin cleavage sites within the GST-HD fusion proteins are shown in

Fig. 8. As outlined above, the proteins GST-HD20DP and -HD51DP were expressed in *E. coli* and affinity-purified under native conditions. They were then digested overnight with trypsin or faxtor Xa protease to promote the formation of polyglutamine-containing huntingtin aggregates. Fig. 9A shows an immunoblot of a cellulose acetate membrane to which the native GST-HD20DP and -HD51DP proteins and their factor Xa and trypsin cleavage products have been applied.

To monitor the in vitro formation of polyglutamine-containing aggregates without the need for a specific antibody, a modified filter retardation assay was developed. In this assay, streptavidin-conjugated alkaline phosphatase (AP) is used to detect the insoluble protein aggregates retained on the cellulose acetate filter membrane. Streptavidin binds specifically to the biotinylation tag (P. J. Schatz, *Biotechnology* **11**, 1138 (1993)) that has been added C-terminal to the polyglutamine tract in the fusion proteins GST-HD20DPBio and -HD51DPBio (Fig. 7) (see Example 8 for details). Fig. 10A shows that the modified aggregation assay gives results comparable to those obtained with the non-biotinylated fusion proteins in that insoluble aggregates are produced from the trypsin-treated GST-HD51DPBio protein but not from the uncleaved GST-HD51DPBio protein or the corresponding 20 repeat samples. Using either fluorescent (AttoPhos^{TM}) or chemiluminescent (CDP-*Star*^{TM}) substrates for alkaline phosphatase, it is possible to capture and quantify the filter assay results with the Boehringer Lumi-Imager F1 system. With both AP substrates, aggregates formed from as little as 5-10 ng of input GST-HD51DPBio protein were readily detected on the cellulose acetate membrane, and signal intensities increased linearly up to 250 ng of fusion protein applied to the filter (Fig. 10B).

Example 6:

Isolation of amyloid-like protein aggregates from transfected COS-1 cells

To examine whether polyglutamine-containing aggregates are also formed *in vivo,* HD exon 1 proteins with 20, 51 or 93 glutamines (without a GST tag) were expressed in COS-1 cells. Whole cell lysates were prepared, and after centrifugation, the insoluble material was collected and treated with DNaseI and trypsin to lower the

viscosity. The resulting protein mixture was then boiled in SDS and analyzed using the dot-blot filter retardation assay (see Example 8). In more detail, the following experimental protocol was carried out:

COS-1 cells were grown in Dulbecco's modified Eagle medium (Gibco BRL) supplemented with 5% (w/v) fetal calf serum (FCS) containing penicillin (5 U/ml) and streptomycin (5 µg/ml), and transfection was performed as described (A. Sittler, D. Devys, C. Weber, and J.-L. Mandel, *Hum. Mol. Genet.* **5**, 95 (1996)).

COS-1 cells transfected with the mammalian expression plasmids pTL1-CAG20, pTL1-CAG51 and pTL1-CAG93 were harvested 48 h after transfection. The cells were washed in ice cold PBS, scraped and pelleted by centrifugation (2000 x g, 10 min, 4°C). Cells were lysed on ice for 30 min in 500 ml lysis buffer [50 m*M* Tris/HCl (pH 8.8), 100 m*M* NaCl, 5 m*M* $MgCl_2$, 0.5% (w/v) NP-40, 1 m*M* EDTA] containing the protease inhibitors PMSF (2 m*M*), leupeptin (10 µl/ml), pepstatin (10 µg/ml), aprotinin (1 µg/ml) and antipain (50 µg/ml). Insoluble material was removed by centrifugation for 5 min at 14000 rpm in a microfuge at 4°C. Pellets containing the insoluble material were resuspended in 100 ml DNase buffer [20 m*M* Tris/HCl (pH 8.0), 15 m*M* $MgCl_2$], and DNase I (Boehringer Mannheim) was added to a final concentration of 0.5 mg/ml followed by incubation at 37°C for 1 h. After DNase treatment the protein concentration was determined by the Dot Metric assay (Geno Technology) using BSA as a standard. Eight µl 1 *M* Tris/HCl (pH 8.4), 1 µl 1% (w/v) SDS, 1 µl 0.2 *M* $CaCl_2$ and 10 µl trypsin (0.25 mg/ml) were then added, and the mixture was incubated for an additional 4 h at 37°C. Digestions were terminated by adjusting the mixtures to 20 m*M* EDTA, 2% (w/v) SDS and 50 m*M* DTT, followed by heating at 98°C for 5 min.

Fig. 9C shows that insoluble protein aggregates are being formed in transfected COS cells expressing the HD exon 1 protein with 51 and 93 glutamines but not in COS cells expressing the normal exon 1 allele with 20 glutamines or in the non-transfected control cells. Thus, as observed *in vitro* with purified GST fusion proteins, formation of high molecular weight protein aggregates *in vivo* occurs in a repeat length-dependent way and requires a polyglutamine repeat in the pathological range. In addition, like

the *in vitro* aggregates, the HD exon 1 aggregates formed *in vivo* are resistant to digestion with trypsin as well as to boiling in 2% (w/v) SDS.

Example 7:

Isolation of amyloid-like protein aggregates from Alzheimer`s disease brain

It has been shown that the neurodegenerative disorder Alzheimer's disease (AD) is caused by the the formation of β-amyloids and neurofibrillar tangles (NFTs) mainly occuring in the neocortex, hippocampus and amygdala (K. Beyreuther, and C.L. Masters, *Nature* **383**, 476 (1996)). To determine whether these structures can be detected by the dot-blot filter retardation assay brain extracts of patients and controls were prepared and analyzed using the anti-Tau, anti-β-amyloid and anti-HD1 antibodies.

Fig. 12 shows that with the anti-Tau and anti-β-amyloid antibodies NFTs and β-amyloids were detected in brain extracts prepared from patients A2 and A3, but not in brain extracts prepared from patient A1 and the controls. Clinical studies revealed that the patients A2 and A3 had Alzheimer's disease with an intermediate and severe intellectual impairment, respectively, whereas patient A1 suffered only from moderate intellectual impairment. This indicates that the results obtained with the filter retardation assay correlate with the severity of the disease. Using the HD1 antibody in the brain extracts prepared from AD patients and controls no aggregated huntingtin protein was detected. However, the antibody reacted with the GST-HD51 protein which was used as a positive control.

Human cerebral cortex (~ 500 mg) was homogenized in 2.5 ml of lysis buffer (0.32 M sucrose, 1 mM $MgCl_2$, 5 mM KH_2PO_4, pH 7.0, 1 mM PMSF) using nine strokes of a glass homogenizer. The homogenat was centrifuged for 15 min at 500 x g to remove the nuclei. The original supernatant was then centrifuged at 93500 x g for 1 h yielding a membrane pellet. The pellet was dissolved in 2 - 5 ml 100 mM Tris-HCl (pH 8), 0.5% SDS and trypsin (Boehringer Mannheim, sequencing grade) was added to a final concentation of 0.05 mg/ml followed by incubation at 37°C overnight. Digestions

were terminated by adjusting the mixtures to 2% SDS and 50 mM DTT, followed by heating at 98°C for 5 min. The mixture was centrifuged for 1 h at 110000 x g and the resulting pellet was resuspended in 100 µl of water. Aliquots (2-10 µl) were then used for the analysis with the dot-blot filter retardation assay.

Example 8:

Dot-blot filter retardation assay

The filter assay used to detect polyglutamine-containing huntingtin protein aggregates has been described (hereinabove and in E. Scherzinger, R. Lurz, M. Trumaine, L. Margiarini, B. Hollenbach, R. Hasenbank, G. P. Bates, S. W. Davies, H. Lehrach, and E. E. Wanker, *Cell* **90**, 549 (1997)). Denatured and reduced protein samples were prepared as described above, and aliquots corresponding to 50-250 ng fusion protein (GST-HD20DP and GST-HD51DP) or 5-30 µg extract protein (pellet fraction) were diluted into 200 µl 0.1% SDS and filtered on a BRL dot blot filtration unit through a cellulose acetate membrane (Schleicher and Schuell, 0.2 µm pore size) that had been preequilibrated with 0.1% SDS. Filters were washed 2 times with 200 µl 0.1% SDS and were then blocked in TBS (100 m*M* Tris/HCl, pH 7.4, 150 m*M* NaCl) containing 3% nonfat dried milk, followed by incubation with the anti-HD1 (1:1000) (see above and E. Scherzinger, R. Lurz, M. Trumaine, L. Margiarini, B. Hollenbach, R. Hasenbank, G. P. Bates, S. W. Davies, H. Lehrach, and E. E. Wanker, *Cell* **90**, 549 (1997), the anti-Tau (Dako, 1:1000) or the anti-β-amyloid antibody (Dako, 1:300). The filters were washed several times in TBS, then incubated with a secondary anti-rabbit or anti-mouse antibody conjugated to horse raddish peroxidase (Sigma, 1:5000) followed by ECL (Amersham) detection. The developed blots were exposed for various times to Kodak X-OMAT film or to a Lumi-Imager (Boehringer Mannheim) to enable quantification of the immunoblots.

For detection and quantification of polyglutamine-containing aggregates generated from the protease-treated fusion proteins GST-HD20DPBio and -HD51DPBio, the biotin/streptavidin-AP detection system was used. Following filtration, the cellulose acetate membranes were incubated with 1% (w/v) BSA in TBS for 1 h at room temperature with gentle agitation on a reciprocal shaker. Membranes were then

incubated for 30 min with streptavidin-alkaline phosphatase (Promega) at a 1:1000 dilution in TBS containing 1% BSA, washed 3 times in TBS containing 0.1% (v/v) Tween 20 and 3 times in TBS, and finally incubated for 3 min with either the fluorescent alkaline phosphatase substrate AttoPhos™ or the chloro-substituted 1,2-dioxetane chemiluminescence substrate CDP-*Star*™ (Boehringer Mannheim) in 100 m*M* Tris/HCl, pH 9.0, 100 m*M* NaCl and 1 m*M* $MgCl_2$. Fluorescent and chemiluminescent signals were imaged and quantified with the Boehringer Lumi-Imager F1 system and LumiAnalyst™ software (Boehringer Mannheim).

Example 9:

Microtitre plate filter retardation assay

To process a large number of proteolytic digestion reactions in parallel, a microtitre plate filter retardation assay was developed. In this assay a 96-well microtitre plate containing a cellulose acetate membrane with a pore size of 0.45 mm (Whatman Polyfiltronics) was used for the retention of polyglutamine-containing protein aggregates.

The following experimental protocol was employed:

First, 15 µl GST fusion protein solution (200 µg/ml GST-HD51DPBio or GST-HD20DPBio in buffer P [20 m*M* Tris/HCl (pH 8.0), 150 m*M* NaCl]) and 15 µl trypsin solution (10 µg/ml trypsin (Boehringer Mannheim, sequencing grade) in buffer P) were combined in a 96-well Thermo-Fast®96 tube plate (Advanced Biotechnologies LTD) using a multi channel pipette (Eppendorf), and the microtitre plate was incubated for 16 hours at 37°C. Then 30 µl SDS/DTTsolution (4% SDS, 100 m*M* DTT in buffer P) were added to each well, the plate was sealed with a microtitre plate sealer (Biostat LTD) and the plate was heated in a 96-well MasterCycler (Eppendorf-Netheler-Hinz) for 5 min at 98°C.

The sealing was removed and 50 µl of the reaction mix were transferred into each well of a new 96-well microtitre plate containing a 0.45 µm cellulose acetate

membrane, pre-equilibrated with 0.1% (w/v) SDS, using a multi channel pipette. For equilibration of the cellulose acetate membrane, the microtitre plate was placed into the QIAvac Manifold-96 (Qiagen) and 200 µl 0.1% SDS was pipetted into each well of the plate. Vacuum was then applied until the SDS solution had passed through the filter. Prior to addition of the protein solution, each well of the filter plate was preloaded with an additional 200 µl of 0.1% SDS. The diluted protein solution was then filtered through the membrane by applying vacuum.

The filterplate was washed with 2 x 200 µl 0.1% SDS and 2 x 200 ml TBS (100 m*M* Tris/HCl (pH 7.4), 150 m*M* NaCl). Vacuum was used to remove wash solutions from the membrane. 200 µl 0.2% (w/v) BSA in TBS were pipetted into each well of the filterplate, and the plate was incubated for 1 h at room temperature (RT) (blocking). Blocking buffer was removed by pipetting.

Next, 200 µl streptavidin alkaline phosphatase (1:1000, Promega) in 0.2% (w/v) BSA/TBS were added to each sample, and the filterplate was incubated for 1 h at RT. Streptavidin AP buffer was removed by pipetting. The filterplate was washed with 3 x 200 µl TTBS [100 m*M* Tris/HCl (pH 7.4), 150 m*M* NaCl, 0.1% (v/v) Tween 20] and 3 x 200 µl TBS. Vacuum was used to remove wash solutions.

200 µl detection buffer (50 m*M* Tris/HCl (pH 9.0), 500 m*M* NaCl, 1 m*M* Mg Cl_2) were added to each sample, incubated for 1 min and vacuum was applied to remove the buffer. 200 µl Attophos™ (10 m*M* AttoPhos™) in detection buffer were pipetted into each well of the filterplate, incubated for 1 h at RT, vacuum was applied to remove the buffer, and the fluorescence emission of each well was measured with the CytoFluor®4000 (Perseptive Biosystems) at 485+/-20 (excitation) and 530 +/-25 (emission). Finally, the resultant images were analysed with CytoFluor 4.1 software and MS Excel 7.0.

As expected from the text set of experiments, using fusions of GST and the full-length HD exon 1 protein, only the cleavage products of GST-HD51DP were retained by the filter and were detected by the huntingtin-specific antibody HD1, indicating the

formation of high molecular weight HD51DP aggregates from this fusion protein. Scanning electron microscopy of the material retained on the surface of the membrane revealed bunches of long fibrils or filaments (Fig. 9B), which were not detected after filtration of the uncleaved GST-HD51DP preparation or the protease-treated GST-HD20DP preparation. These results indicate that an elongated polyglutamine sequence but not the proline rich region in the HD exon 1 protein is necessary for the formation of high molecular weight protein aggregates *in vitro*.

Essentially, the same results as with the dot blot filter retardation assay were obtained when the fusion proteins GST-HD20DPBio and -HD51DPBio were analysed with the microtitre plate filter retardation assay, indicating that this assay can be used for the high throughput isolation of chemical compounds from chemical libraries (Fig. 11A and B).

References

Bates, G. P., Mangiarini, L., Mahal, A. and Davies, S. W. (1997). Transgenic models of Huntingtons disease. Hum. Mol. Genet. *6,* 1633-1637.

Booth, D. R., Stunde, M., Bellotti, V., Robinson, C. V., Hutchinson, W. L., Fraser, P. E., Hawkins, P. N., Dobson, C. M., Radford, S. E., Blake, C. C. F., and Pepys, M. B. (1997). Instability, unfolding and aggregation of human lysozyme variants underlying amyloid fibrillogenesis. Nature *385*, 787-793.

Burke, J. R., Enghild, J. J., Martin, M. E., Jou, Y.-S., Myers, R. M., Roses, A. D., Vance, J. M., and Strittmatter, W. J. (1996). Huntingtin and DRPLA proteins selectively interact with the enzyme GAPDH. Nature Med. *2*, 347-350.

Caputo, C. B., Fraser, P. E., Sobel, I. E., and Krischner, D. A. (1992). Amyloid-like properties of a synthetic peptide corresponding to the carboxy terminus of b-amyloid protein precursor. Arch. Bioch. Biophys. *292*, 199-205.

Caughey, B., and Chesebro, B. (1997). Prion protein and the transmissible spongiform encephalopaties. Trends Cell Biol. *7*, 56-62.

Davies, S. W., Trumaine, M., Cozens, B. A., DiFiglia, M., Sharp, A. H., Ross, C. A., Scherzinger, E., Wanker, E. E., Mangiarini, L., and Bates, G. P. (1997). Formation of neuronal intranuclear inclusions (NII) underlies the neurological dysfunction in mice transgenic for the HD mutation. Cell 90,537-548.

de Rooij, K. E., Dorsman, J. C., Smoor, M. A., T., d. D. J., and van Ommen, G.-J. (1996). Subcellular localisation of the Huntington's disease gene product in cell lines by immunofluorescence and biochemical subcellular fractionation. Hum. Mol. Genet. *5*, 1093-1099.

DiFiglia, M., Sapp, E., Chase, K., Schwarz, C., Meloni, A., Young, C., Martin, E., Vonstattel, J.-P., Carraway, R., Reeves, S. A., Boyce, F. M., and Aronin, N. (1995). Huntingtin is a cytoplasmic protein associated with vesicles in human and rat brain neurons. Neuron *14*, 1075-1081.

Duyao, M. P., Auerbach, A. A., Ryan, A., Persichetti, F., Barnes, G. T., McNeil, S. M., Ge, P., Vonstattel, J.-P., Gusella, J. F., Joyner, A. L., and MacDonald, M. E. (1995). Inactivation of the mouse Huntington's disease gene homolog *Hdh*. Science *269*, 407-410.

Glenner, G. G. (1980). Amyloid deposits and amyloidosis. N. Engl. J. Med. *302*, 1283-1292, 1333-1343.

Goldberg, Y. P., Nicholson, D. W., Rasper, D. M., Kalchman, M. A., Koide, H. B., Graham, R. K., Bromm, M., Kazemi-Esfarjani, P., Thornberry, N. A., Vaillancourt, J. P., and Hayden, M. R. (1996). Cleavage of huntingtin by apopain, a proapoptotic cysteine protease, is modulated by the polyglutamine tract. Nature Genet. *13*, 442-449.

Gutekunst, C.-A., Levey, A. I., Heilman, C. J., Whaley, W. L., Yi, H., Nash, N. R., Rees, H. D., Madden, J. J., and Hersch, S. M. (1995). Identification and localization of huntingtin in brain and human lymphoblastoid cell lines with anti-fusion protein antibodies. Proc. Natl. Acad. Sci. USA *92*, 8710-8714.

Harper, P. S. (1991). Huntington's disease, 22 Edition, P. S. Harper, ed. (London: W.B. Saunders Co, Ltd).

HDCRG (1993). A novel gene containing a trinucleotide repeat that is unstable on Huntington's disease chromosomes. Cell *72*, 971-983.

Hoogeveen, A. T., Willemsen, R., Meyer, N., de Rooij, K. E., Roos, R. A. C., van Ommen, G.-J. B., and Galjaard, H. (1993). Characterisation and localisation of the Huntington disease gene product. Hum. Mol. Genet. *2*, 2069-2073.

Ikeda, H., Yamaguchi, M., Sugai, S., Aze, Y., Narumiya, S., and Kakizuka, A. (1996). Expanded polyglutamine in the Machado-Joseph disease protein induces cell death *in vitro* and *in vivo*. Nature Genet. *13*, 196-202.

Jarrett, J. T., and Lansburry, P. T. (1993). Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie? Cell *73*, 1055-1058.

Kalchman, M. A., Graham, R. K., Xia, G., Koide, H. B., Hodgson, J. G., Graham, K. C., Goldberg, Y. P., Gietz, R. D., Pickart, C. M., and Hayden, M. R. (1996). Huntingtin is ubiquinated and interacts with a specific ubiquitin-conjugating enzyme. J. Biol. Chem. *271*, 19385-19394.

Kalchman, M. A., Koide, H. B., McCutcheon, K., Graham, R. K., Nichol, K., Nishiyama, K., Kazemi-Esfariani, P., Lynn, F. C., Wellington, C., Metzler, M., Goldberg, Y. P., Kanazawa, I., Gietz, R. D., and Hayden, M. R. (1997). *HIP1*, a human homologue of *S. cerevisiae* Sla2p, interacts with mambrane-associated huntingtin in the brain. Nature Genet. *16*, 44-53.

Li, X.-J., Li, S.-H., Sharp, A. H., Nucifora, F. C., Schilling, G., Lanahan, A., Worley, P., Snyder, S. H., and Ross, C. A. (1995). A huntingtin-associated protein enriched in brain with implications for pathology. Nature *378*, 398-402.

Lim, K., Ho, J. X., Keeling, K., Gilliland, G. L., JI, X., Rüker, F., and Carter, D. C. (1994). Three-dimensional structure of Schistosoma japonicum glutamine S-transferase fused with a six-amino acid conserved neutralizing epitope of gp41 from HIV. Prot. Sci. *3*, 2233-2244.

Mangiarini, L., Sathasivam, K., Seller, M., Cozens, B., Harper, A., Hetherington, C., Lawton, M., Trottier, Y., Lehrach, H., Davies, S. W., and Bates, G. P. (1996). Exon 1 of the Huntington's disease gene containing a highly expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice. Cell *87*, 493-506.

Onodera, O., Roses, A. D., Tsuji, S., Vance, J. M., Stritmatter, W. J., and Burke, J. R. (1996). Toxicity of expanded polyglutamine-domain proteins in *Escherichia coli*. FEBS Lett. *399*, 135-139.

Perutz, M. F. (1996). Gulutamine repeats and inherited neurodegenerative diseases: molecular aspects. Curr. Opin. Struct. Biol. *6*, 848-858.

Perutz, M. F., Johnston, T., Suzuki, M., and Finch, J. T. (1994). Glutamine repeats as polar zippers: their possible role in neurodegenerative diseases. Proc. Natl. Acad. Sci. USA *91*, 5355-5358.

Portera-Cailliau, C., Hedreen, J. C., Price, D. L., and Koliatsos, V. E. (1995). Evidence of apoptotic cell death in Huntington disease and excitotoxic animal models. J. Neurosci. *15*, 3775-3787.

Prusiner, S. B., Kinley, M. P. M., Bowman, K. A., Bolton, D. C., Bendheim, P. E., Groth, D. F., and Glenner, G. G. (1983). Scrapie prions aggregate to form amyloid-like birefingent rods. Cell *35*, 349-358.

Roizin, L., Stellar, S., and Liu, J. C. (1979). Neuronal nuclear-cytoplasmic changes in Huntington's Chorea: Electron microcope investigations. Adv. Neurol. *23*, 95-122.

Roos, R. A. C., and Bots, G. T. A. M. (1983). Nuclear membrane indentations in Huntington's chorea. J. Neurol. Sci. *61*, 37-47.

Ross, C. A. (1995). When more is less: pathogenesis of glutamine repeat neurodegenerative diseases. Neuron *15*, 493-496.

Rubinsztein, D. C., Leggo, J., Coles, R., Almqvist, E., Biancalana, V., Cassiman, J.-J., Chotai, K., Connarty, M., Crauford, D., Curtis, A., Curtis, D., Davidson, M. J., Differ, A.-M., Dode, C., Dodge, A., Frontali, M., Ranen, N. G., Stine, O. C., Sherr, M., Abbott, M. H., Franz, M. L., Graham, C. A., Harper, P. S., Hedreen, J. C., Jackson, A., Kaplan, J.-C., Losekoot, M., MacMillan, J. C., Morrison, P., Trottier, Y., Novelletto, A., Simpson, S. A., Theilmann, J., Whittaker, J. L., Folstein, S. E., Ross, C. A., and Hayden, M. R. (1996). Phenotypic characterisation of individuals with 30-40 CAG repeats in the Huntington's disease (HD) gene reveals HD cases with 36 repeats and apparently normal elderly individuals with 36-39 repeats. Am. J. Hum. Genet. *59*, 16-22.

Sathasivam, K., Amaechi, I., Mangiarini, L., and Bates, G. P. (1997). Identification of an HD patient with a (CAG)180 repeat expansion and the propagation of highly expanded CAG repeats in lambda phage. Hum Genet. *99*, 692-695.

Sharp, A. H., Loev, S. J., Schilling, G., Li, S.-H., Li, X.-J., Bao, J., Wagster, M. V., Kotzuk, J. A., Steiner, J. P., Lo, A., Hedreen, J., Sisodia, S., Snyder, S. H., Dawson, T. M., Ryugo, D. K., and Ross, C. A. (1995). Widespread expression of Huntington's disease gene (IT15) protein product. Neuron *14*, 1065-1074.

Smith, D. B., and Johnson, K. S. (1988). Single-step purification of peptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene *67*, 31-40.

Stott, K., Blackburn, J. M., Butler, P. J. G., and Perutz, M. (1995). Incorporation of glutamine repeats makes protein oligomerize: implications for neurodegenerative diseases. Proc. Natl. Acad. Sci. USA *92*, 6509-6513.

Tellez-Nagel, I., Johnson, B., and Terry, R. D. (1974). Studies on brain biopsies of patients with Huntington's chorea. J. Neurocyt. *3*, 308-332.

Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA *76*, 4350-4354.

Trottier, Y., Devys, D., Imbert, G., Sandou, F., An, I., Lutz, Y., Weber, C., Agid, Y., Hirsch, E. C., and Mandel, J.-L. (1995a). Cellular localisation of the Huntington's disease protein and discrimination of the normal and mutated forms. Nature Genet. *10*, 104-110.

Trottier, Y., Lutz, Y., Stevanin, G., Imbert, G., Devys, D., Cancel, G., Sandou, F., Weber, C., David, G., Tora, L., Agid, Y., Brice, A., and Mandel, J.-L. (1995b). Polyglutamine expansion as a pathological epitope in Huntington's disease and four dominant cerebellar ataxias. Nature *378*, 403-406.

Vonsattel, J.-P., Myers, R. H., Stevens, T. J., Ferrante, R. J., Bird, E. D., and Richardson, E. P. (1985). Neuropathological classification of Huntington's disease. J. Neuropath. Exp. Neurol. *44*, 559-577.

Wanker, E. E., Rovira, C., Scherzinger, E., Hasenbank, R., Walter, S., Tait, D., Colicelli, J., and Lehrach, H. (1997). HIP-1: A huntingtin interacting protein isolated by the yeast two-hybrid system. Hum. Mol. Genet. *6*, 487-495.

CLAIMS

1. A method of detecting the presence of detergent- or urea-insoluble amyloid-like fibrils or protein aggregates on a filter comprising the following steps:
   (a) contacting said filter with material suspected to comprise said fibrils or aggregates; and
   (b) detecting whether said fibrils or aggregates are retained on said filter.

2. The method of claim 1 wherein said amyloid-like fibrils or protein aggregates are indicative of a disease.

3. The method of claim 2 wherein said disease is a human disease.

4. The method of claim 2 or 3 wherein said disease is associated with a polyglutamine expansion.

5. The method of any one of claims 2 to 4 wherein said disease is Huntington's disease, spinal and bulbar muscular atrophy, dentarorubral pallidoluysian atrophy, spinocerebellar ataxia type-1, -2, -3, -6 or -7, Alzheimer disease, BSE, primary systemic amyloidosis, secondary systemic amyloidosis, senile systemic amyloidosis, familial amyloid polyneuropathy I, hereditary cerebral amyloid angiopathy, hemodialysis-related amyloidosis, familial amyloid polyneuropathy III, Finnish hereditary systemic amyloidosis, type II diabetes, medullary carcinoma of the thyroid, spongiform encephalopathies: Kuru, Gerstmann- Sträussler-Scheinker syndrome (GSS), familial insomnia, scrapie, atrial amyloidosis, hereditary non-neuropathic systemic amyloidosis, injection-localized amyloidosis, hereditary renal amyloidosis, or Parkinson's disease.

6. The method of any one of claims 1 to 5 wherein said filter is comprised of material with low protein adsorption.

7. The method of claim 6 wherein said material with low protein adsorption is cellulose acetate.

8. The method of any one of claims 1 to 7 wherein, prior to step (b), the following step is carried out:
(b') washing said filter so as to remove detergent- or urea-soluble material.

9. The method of any one of claims 1 to 8 wherein detergent- or urea-soluble material is simultaneously with or subsequent to step (a), sucked through said filter.

10. The method of any one of claims 1 to 9 wherein detection in step (b) is effected by an antibody, or (poly)peptide, preferably a tag or an enzyme, or a fragment or derivative thereof or a chemical reagent that specifically binds to said fibrils or aggregates.

11. The method of any one of claims 1 to 9 wherein detection in step (b) is effected by electron microscopy, electron scanning microscopy, fluorescence or chemiluminescence.

12. The method of any one of claims 1 to 11 wherein said material is derived from tissues or cells of bacteria, yeast, fungi, plants, insects, animals, preferably mammals, humans, from a transgenic animal or a transgenic plant.

13. The method of any one of claims 1 to 11 further comprising the following steps prior to step (a):
(a') incubating a fusion protein comprising a (poly)peptide that enhances solubility and/or prevents aggregation of said fusion protein, an amyloidogenic (poly)peptide that has the ability to self-assemble into amyloid-like fibrils or protein aggregates when released from said fusion protein and a cleavable site that separates the above-mentioned components of the fusion protein in the presence of a suspected inhibitor of amyloid-like fibril or protein aggregate formation; and

(a") simultaneously with or after step (a'), further incubating with a compound that induces cleavage at said cleavage site.

14. The method of claim 13 wherein said cleavable site is an enzymatically cleavable site or a chemically cleavable site or a site cleavable by intein self-cleavage in the presence of thiols.

15. The method of claim 13 or 14 further comprising, prior to step (b) and after step (a"):
(a"') incubation with an inhibitor of said compound that induces cleavage.

16. The method of any one of claims 13 to 15 wherein said amyloidogenic (poly)peptide comprises a polyglutamine expansion.

17. The method of any one of claims 4 to 16 wherein said polyglutamine expansion comprises at least 35, preferably at least 41, more preferably at least 48 and most preferably at least 51 glutamines.

18. The method of any one of claims 1 to 17 wherein said contacting is effected by dotting, spotting or pipetting said material onto said filter.

19. The method of any one of claims 1 to 18 wherein said filter is a filter membrane.

20. The method of any one of claims 1 to 19 wherein said detergent is SDS or Triton X-100.

21. An inhibitor identified by the method of any one of claims 13 to 19.

22. The inhibitor of claim 21 which is an antibody or a derivative or functional fragment thereof, a peptide or a chemical reagent.

23. A pharmaceutical composition comprising the inhibitor of claim 21 to 22 and a pharmaceutically acceptable carrier and/or diluent.

24. A diagnostic composition comprising
(i) a fusion protein as defined in any one of the preceding claims.

25. The diagnostic composition of claim 24 further comprising

(ii) a filter as defined in any one of the preceding claims optionally or preferably contained in a microtiter plate; and optionally

(iii) a compound that induces cleavage as defined in any one of the preceding claims; and optionally

(iv) an inhibitor of said compound of (c); and optionally

(v) suitable buffer solutions.

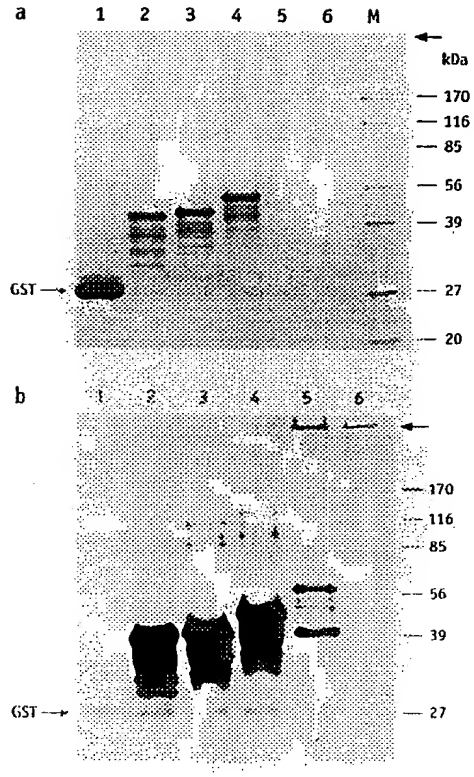

a
1 2 3 4 5 6 M
kDa
170
116
85
56
39
GST
27
20
b
1 2 3 4 5 6
170
116
85
56
39
GST
27

Figure 1
1/12

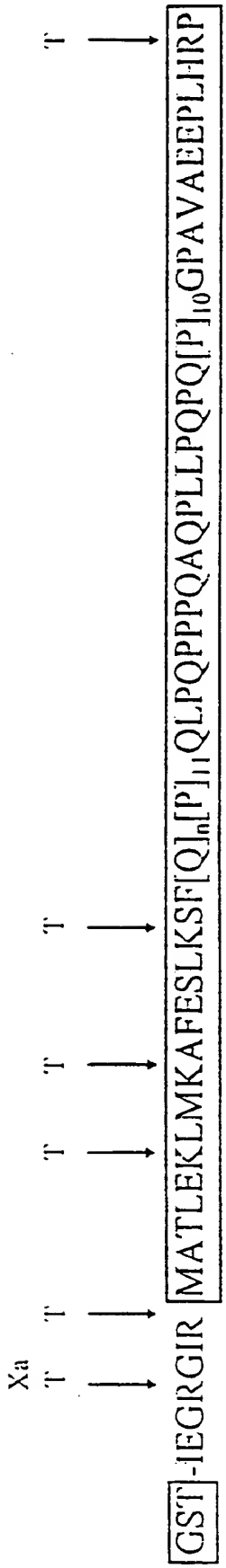

T
T
T
T
T
T
Xa
T
GST-IEGRGIR MATLEKLMKAFESLKSF[Q]n[P]11QLPQPPPQAQPLLPQPQ[P]10GPAVAEEPLHRP

Figure 2
2/12

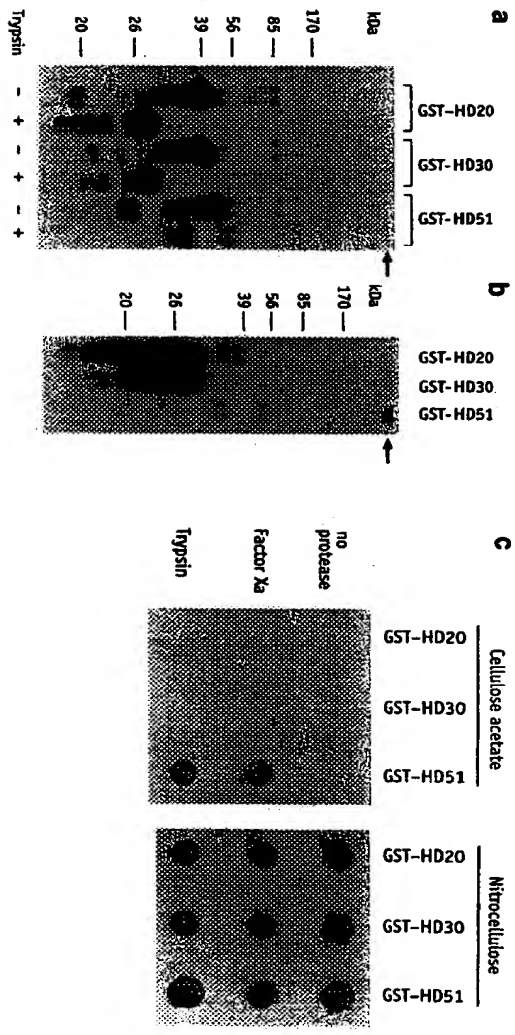

a
kDa
170
85
56
39
26
20
Trypsin
- + - + - +
GST-HD20
GST-HD30
GST-HD51
b
kDa
170
85
56
39
26
20
GST-HD20
GST-HD30
GST-HD51
c
Trypsin
Factor Xa
no protease
GST-HD20
GST-HD30
GST-HD51
Cellulose acetate
GST-HD20
GST-HD30
GST-HD51
Nitrocellulose

Figure 3
3/12

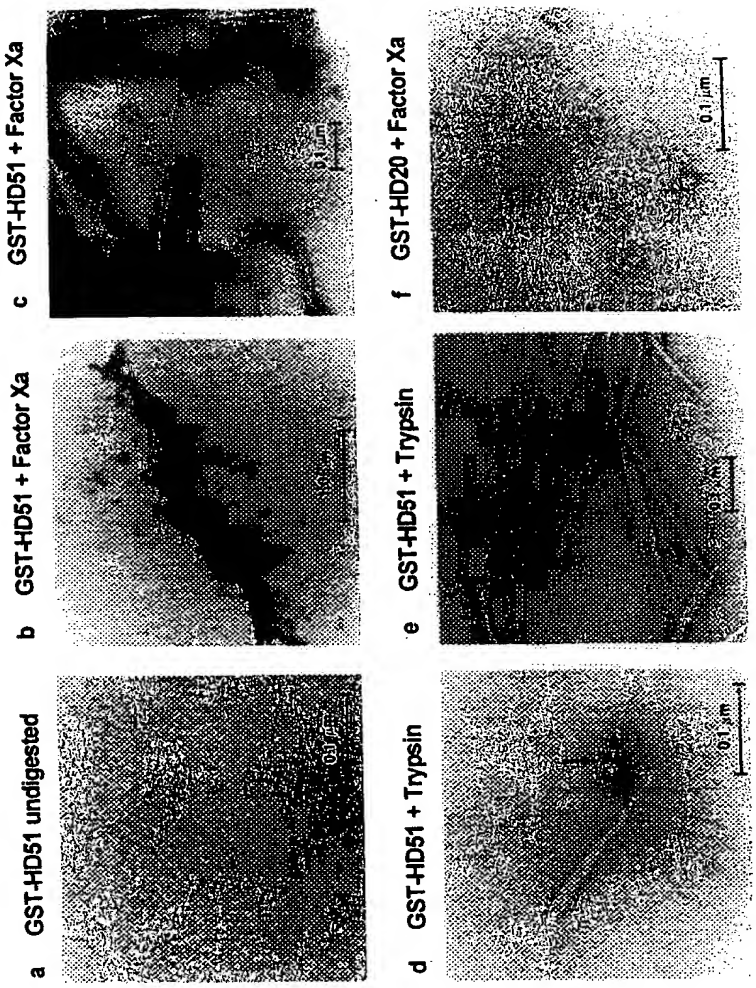

a GST-HD51 undigested
b GST-HD51 + Factor Xa
c GST-HD51 + Factor Xa
d GST-HD51 + Trypsin
e GST-HD51 + Trypsin
f GST-HD20 + Factor Xa
0.1 µm

Figure 4
4/12

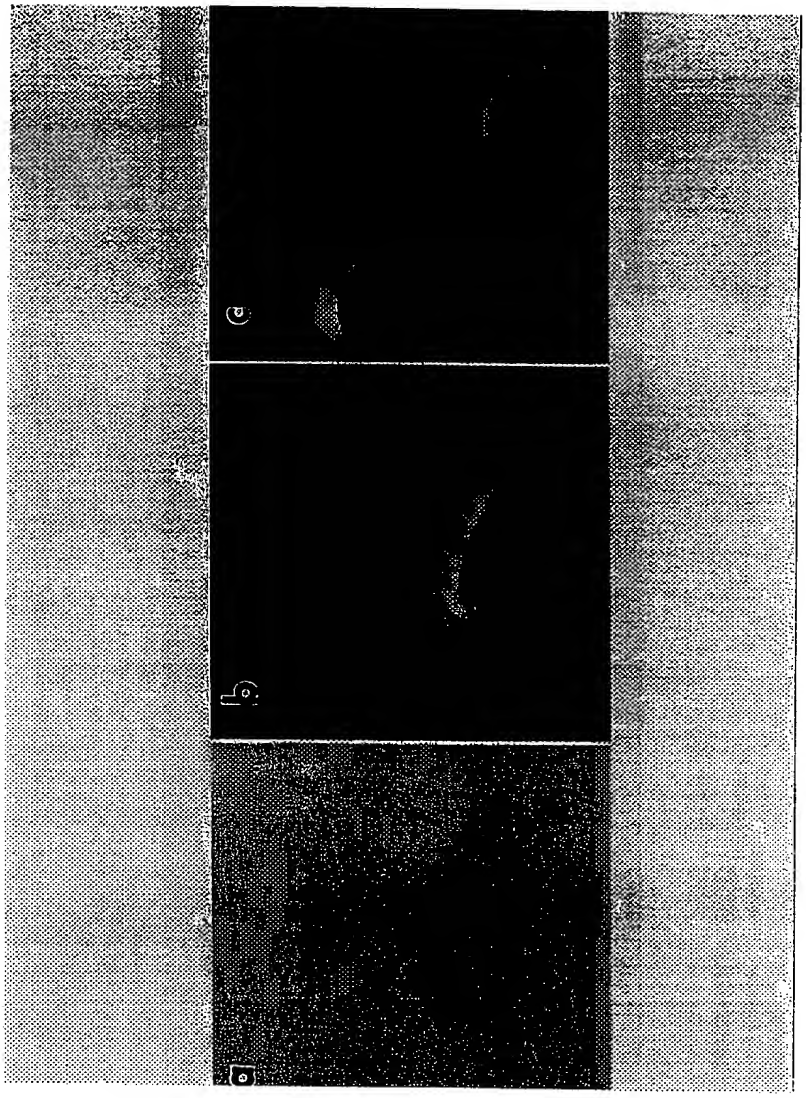


Figure 5

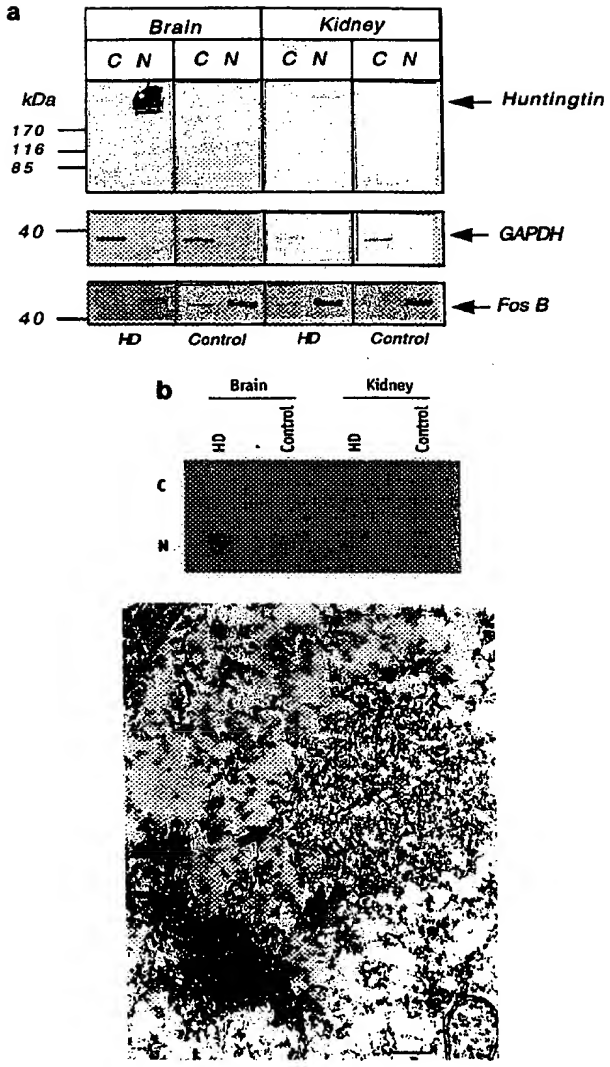

a
Brain
Kidney
C N C N C N C N
kDa
170
116
85
Huntingtin
40
GAPDH
40
Fos B
HD Control HD Control
b
Brain
Kidney
HD Control HD Control
C
N

Figure 6

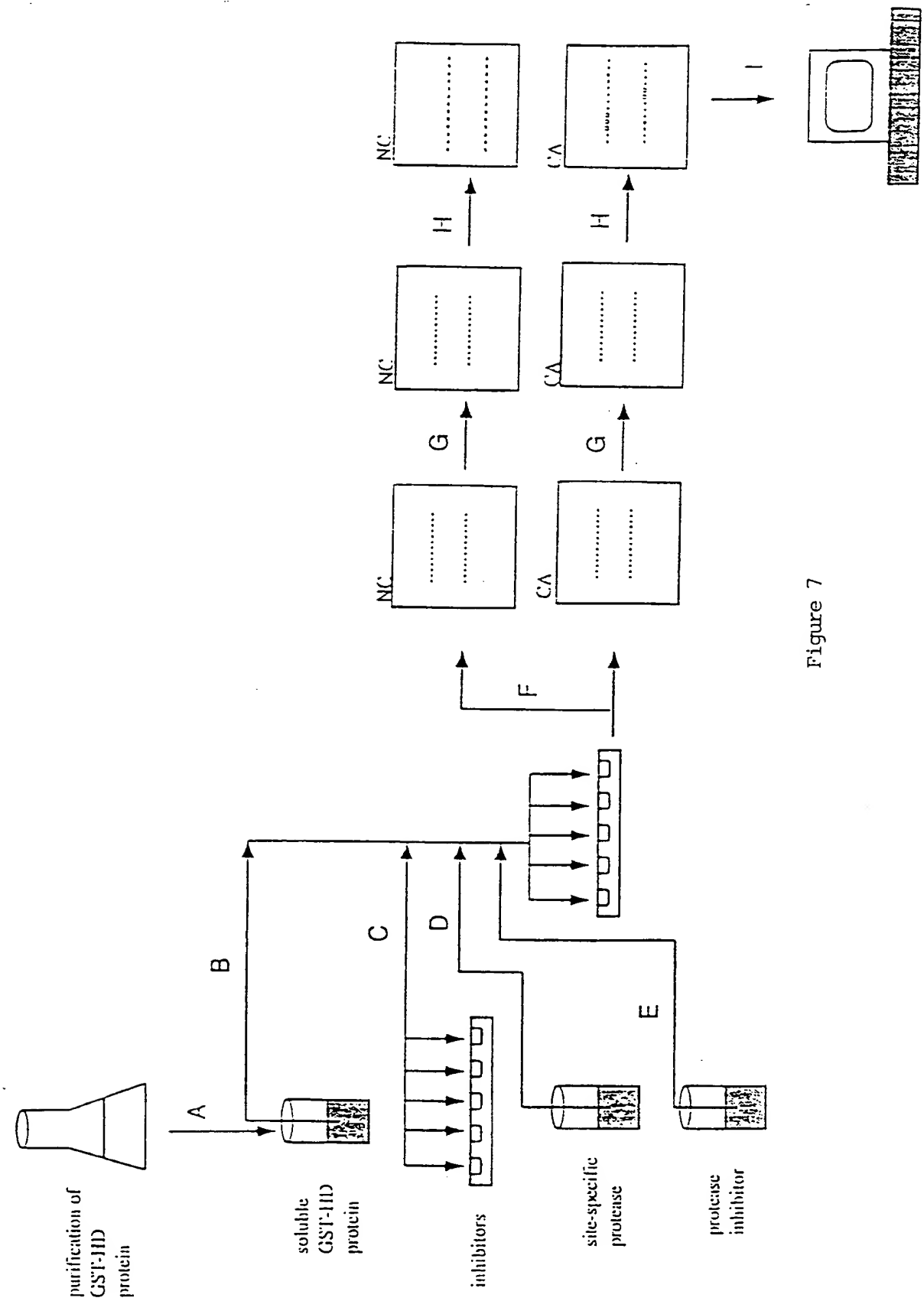

purification of GST-HD protein
A
soluble GST-HD protein
B
inhibitors
C
site-specific protease
D
protease inhibitor
E
F
NC
CA
G
H
I

Figure 7

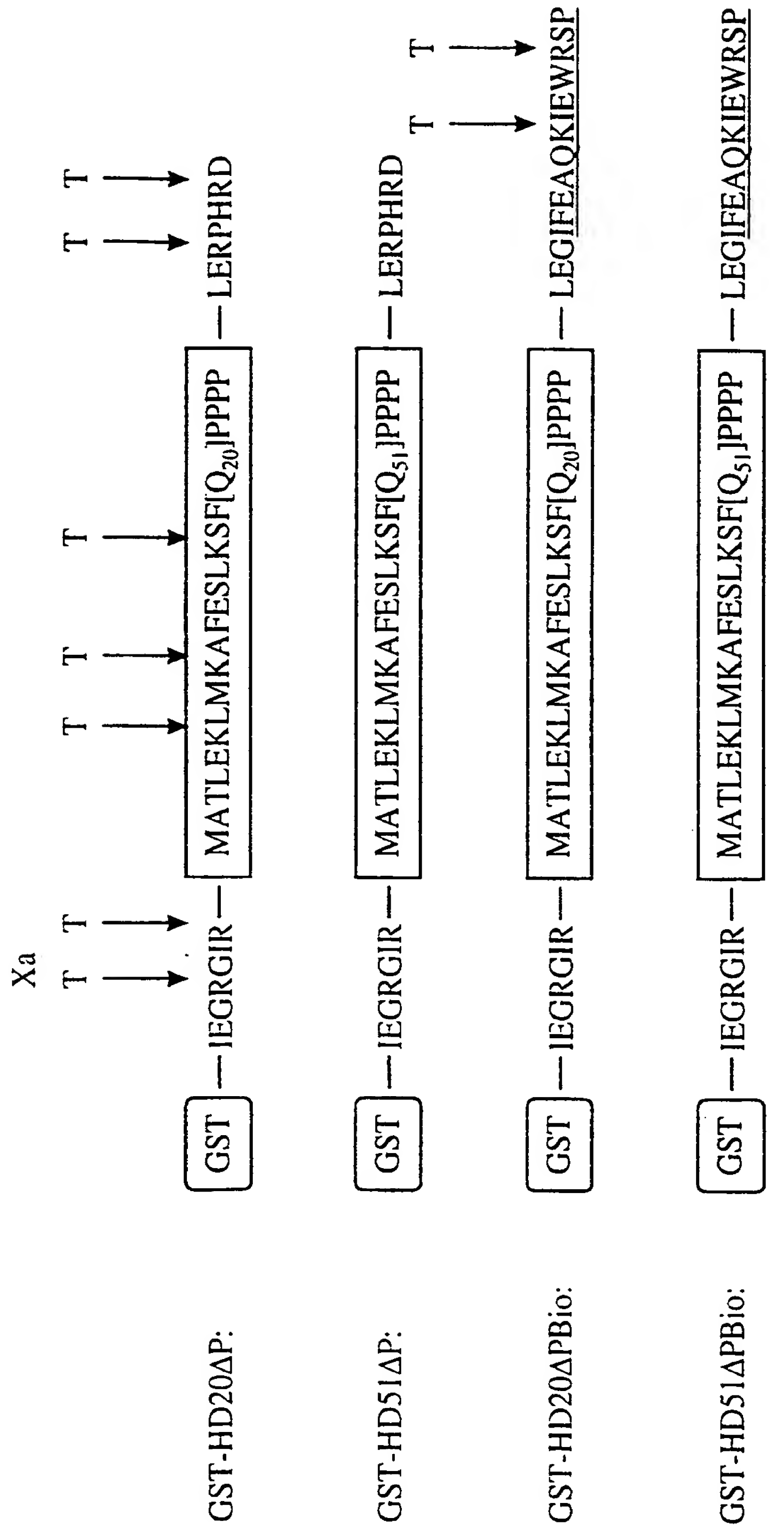

GST-HD20ΔP:
GST-HD51ΔP:
GST-HD20ΔPBio:
GST-HD51ΔPBio:
GST
IEGRGIR
MATLEKLMKAFESLKSF[Q20]PPPP
MATLEKLMKAFESLKSF[Q51]PPPP
LERPHRD
LEGIFEAQKIEWRSP
Xa
T

Figure 8

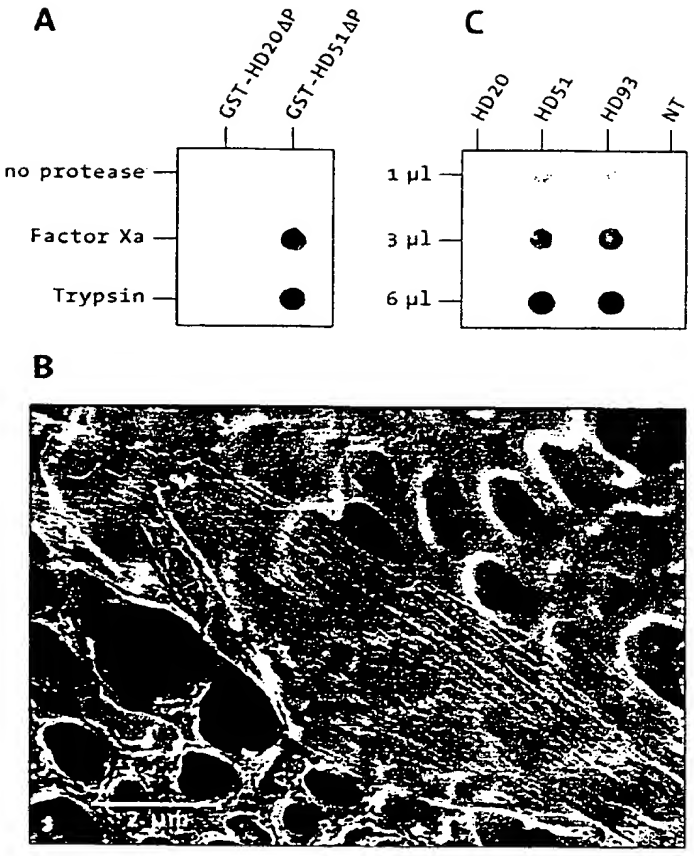
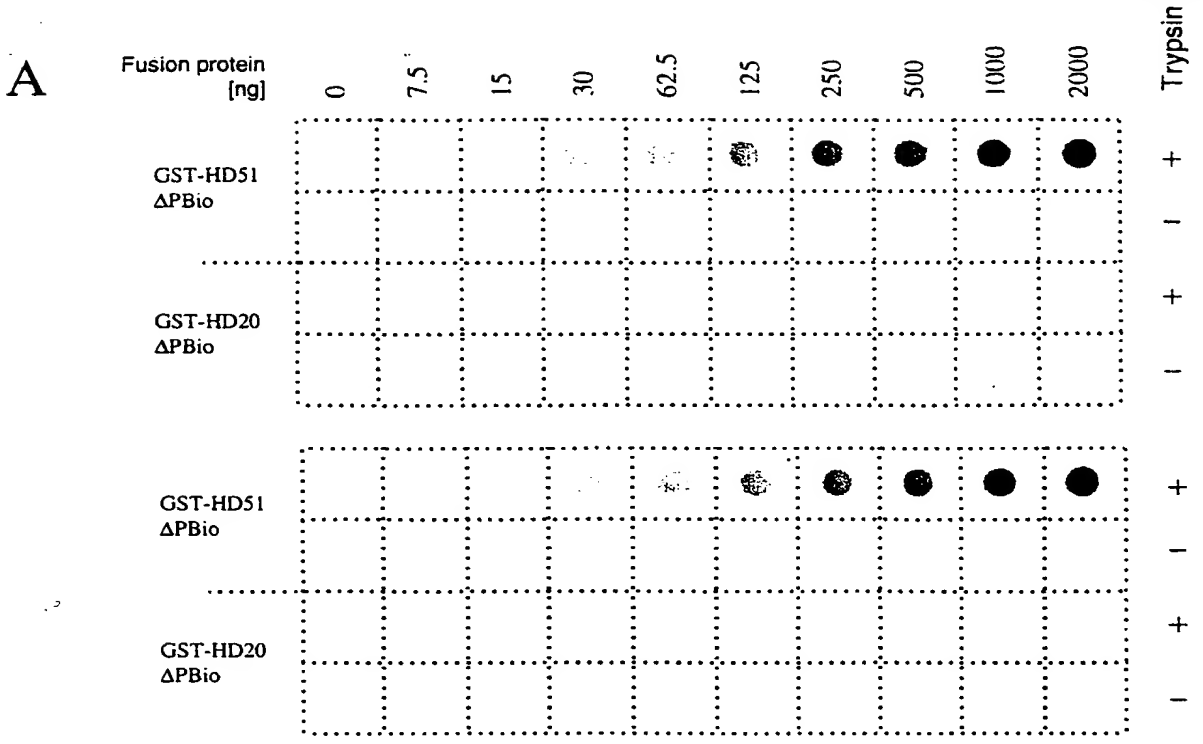

A
GST-HD20ΔP
GST-HD51ΔP
no protease
Factor Xa
Trypsin
C
HD20
HD51
HD93
NT
1 µl
3 µl
6 µl
B

Figure 9



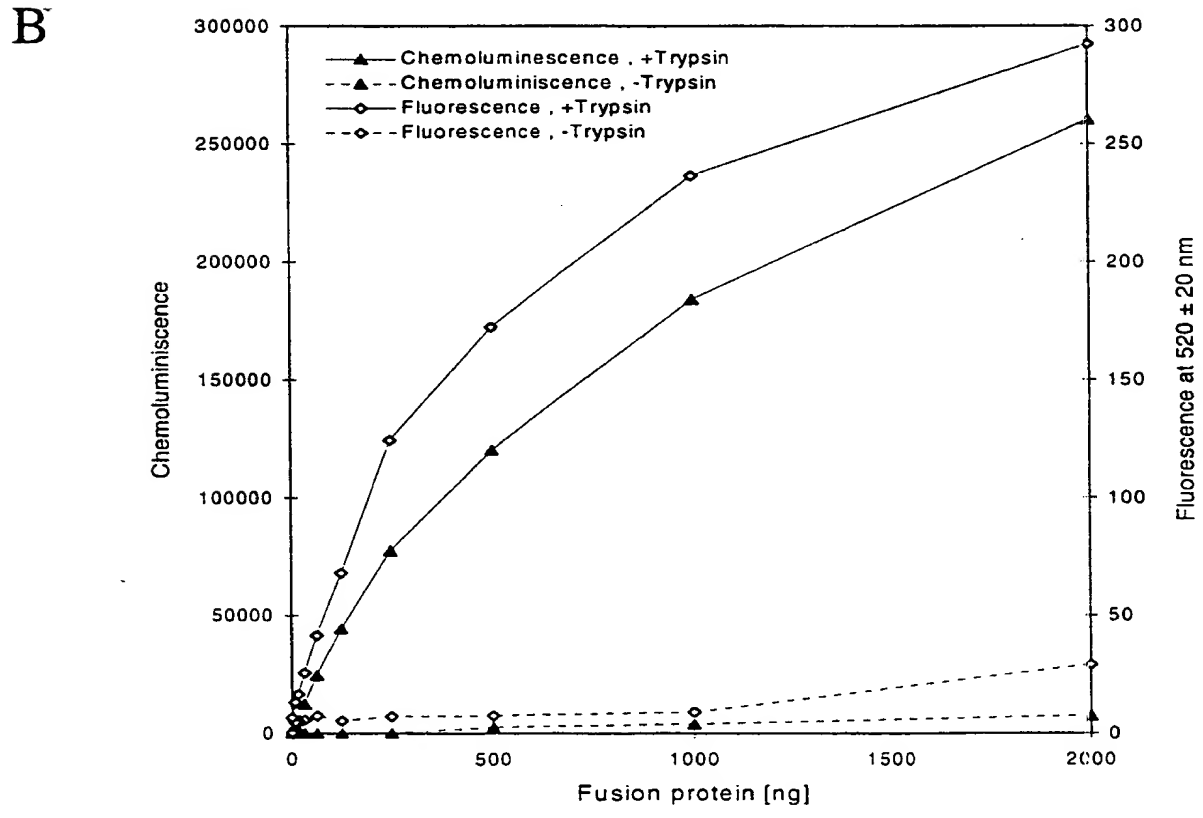
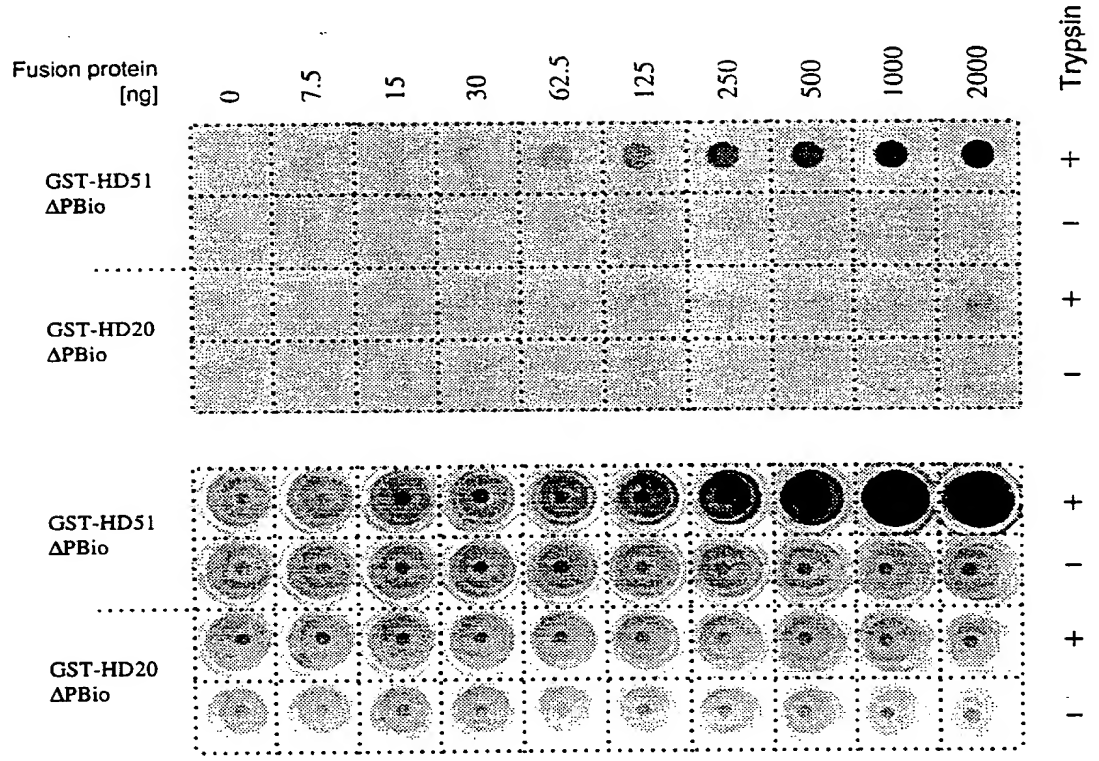


Figure 10
10/12

A


Fusion protein [ng]
0
7.5
15
30
62.5
125
250
500
1000
2000
Trypsin
GST-HD51 ΔPBio
GST-HD20 ΔPBio
GST-HD51 ΔPBio
GST-HD20 ΔPBio
+
−
+
−
+
−
+
−

B

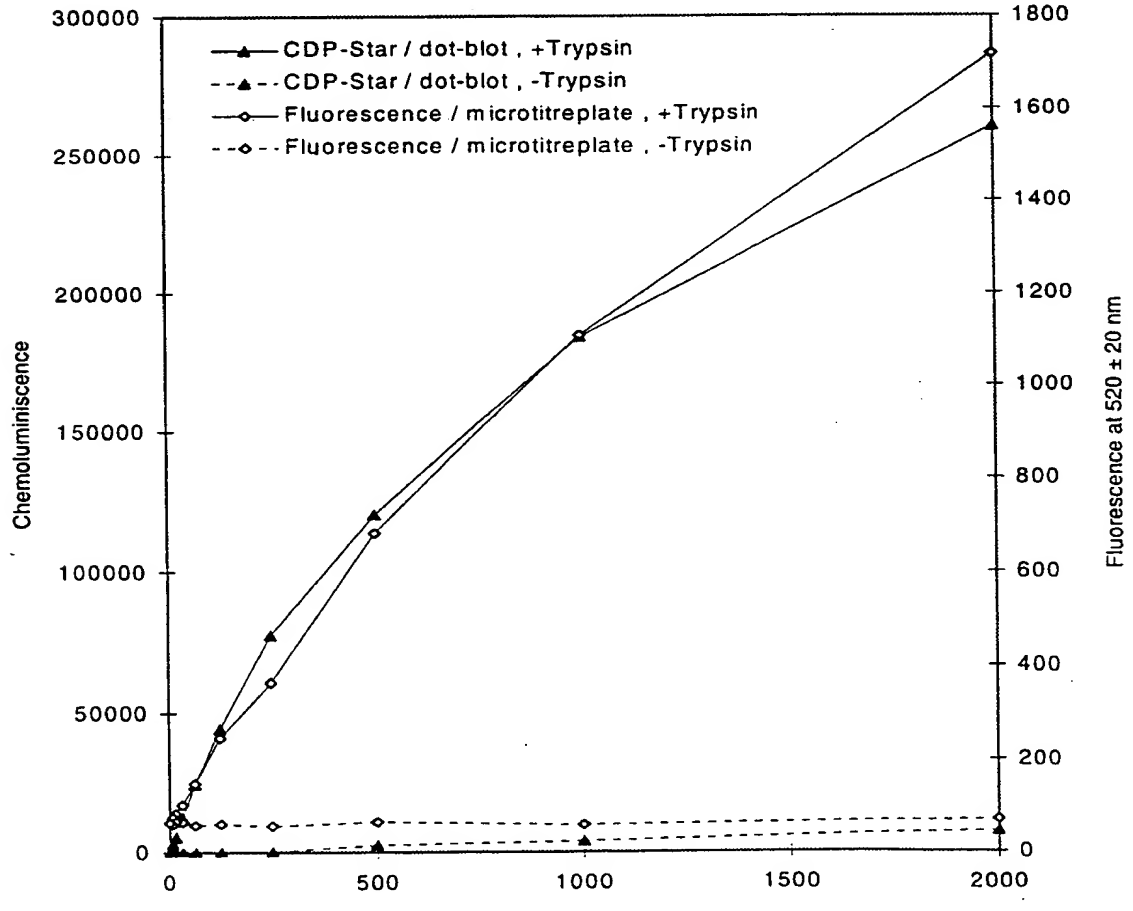

CDP-Star / dot-blot , +Trypsin
CDP-Star / dot-blot , -Trypsin
Fluorescence / microtitreplate , +Trypsin
Fluorescence / microtitreplate , -Trypsin
Chemoluminiscence
Fluorescence at 520 ± 20 nm
300000
250000
200000
150000
100000
50000
0
1800
1600
1400
1200
1000
800
600
400
200
0
0
500
1000
1500
2000

Figure 11

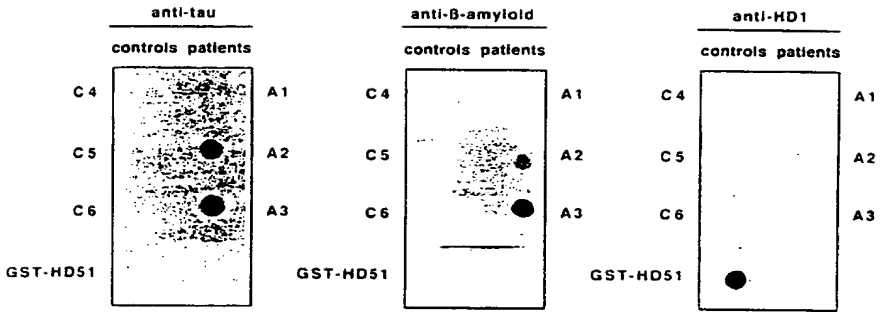


Figure 12
12/12

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/68 A61K35/00 A61K38/00 A61K39/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 206 302 A (CIBA GEIGY AG) 30 December 1986 see page 3 - page 4 ---	1-3,5, 10-12
A,P	EP 0 854 364 A (NARANG HARASH KUMAR) 22 July 1998 see claims; examples ---	1
A	US 5 234 814 A (CARD JOHN P ET AL) 10 August 1993 see column 4 - column 7; example 3 ---	1
A	WO 96 12544 A (GEN HOSPITAL CORP ;TANZI RUDOLPH E (US); BUSH ASHLEY I (US); MOIR) 2 May 1996 see examples --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 April 1999	18/05/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreno, C

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	TATEISHI, J. ET AL: "Removal of causative agent of Creutzfeldt-Jakob disease (CJD) through membrane filtration method." MEMBRANE, vol. 18, no. 6, 1993, pages 357-362, XP002101228 see the whole document	1

1

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : G01N 33/68, A61K 35/00, 38/00, 39/00	A3	(11) International Publication Number: WO 99/06838
		(43) International Publication Date: 11 February 1999 (11.02.99)

(21) International Application Number: PCT/EP98/04810

(22) International Filing Date: 31 July 1998 (31.07.98)

(30) Priority Data:
97113320.2 1 August 1997 (01.08.97) EP

(71) Applicant *(for all designated States except US)*: MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V. [DE/DE]; Berlin (DE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: WANKER, Erich [AT/DE]; Leichhardtstrasse 61, D-14195 Berlin (DE). LEHRACH, Hans [AT/DE]; Lützelsteiner Weg 50, D-14195 Berlin (DE). SCHERZINGER, Eberhard [DE/DE]; Lützelsteiner Weg 52, D-14195 Berlin (DE). BATES, Gillian [GB/GB]; United Medical and Dental Schools, Division of Medical and Molecular Genetics, Guy's Tower, 8th floor, Guy's Hospital, London SE1 9RT (GB).

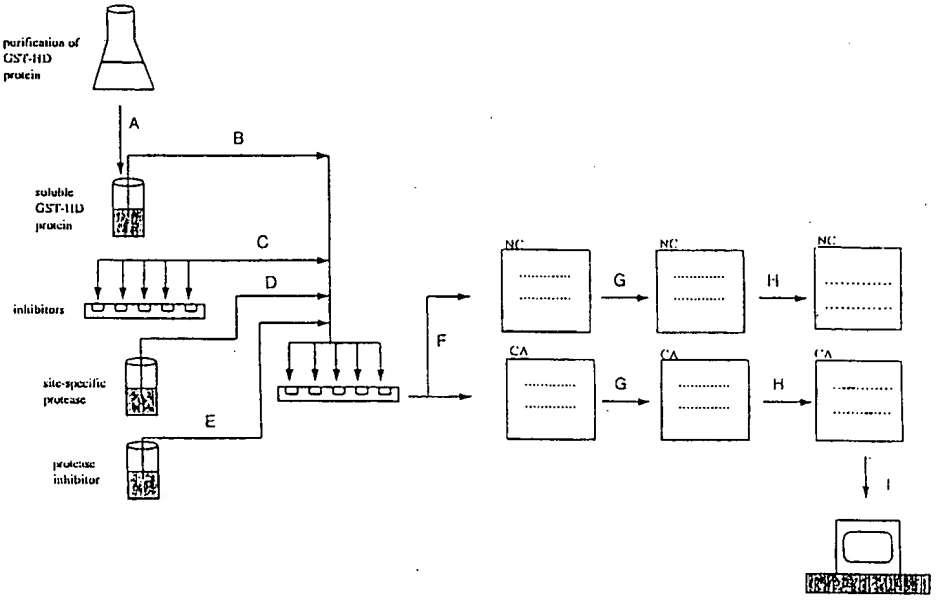
(74) Agent: VOSSIUS & PARTNER; Postfach 86 07 67, D-81634 München (DE).

(81) Designated States: CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
8 July 1999 (08.07.99)

(54) Title: NOVEL METHOD OF DETECTING AMYLOID-LIKE FIBRILS OR PROTEIN AGGREGATES



(57) Abstract

The present invention relates to methods of detecting the presence of detergent- or urea-insoluble amyloid-like fibrils or protein aggregates on filters. Preferably, said fibrils or aggregates are indicative of a disease, preferably of a neurodegenerative disease such as Alzheimer's disease or Huntington's disease. In addition, the present invention relates to inhibitors identified by the method of the invention, to pharmaceutical compositions comprising said inhibitors and to diagnostic compositions useful for the investigation of said amyloid-like fibrils or aggregates.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 98/04810

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0206302 A	30-12-1986	AU 5915186 A	08-01-1987
		DK 297086 A	26-12-1986
		FI 862595 A	26-12-1986
		GR 861603 A	20-10-1986
		JP 62002161 A	08-01-1987
		US 4782014 A	01-11-1988
EP 0854364 A	22-07-1998	NONE	
US 5234814 A	10-08-1993	AU 627046 B	13-08-1992
		AU 5817390 A	07-01-1991
		CA 2017832 A	01-12-1990
		EP 0474765 A	18-03-1992
		JP 4505865 T	15-10-1992
		WO 9015331 A	13-12-1990
WO 9612544 A	02-05-1996	AU 8083094 A	15-05-1996

VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS

PCT

INTERNATIONALER RECHERCHENBERICHT

(Artikel 18 sowie Regeln 43 und 44 PCT)

Aktenzeichen des Anmelders oder Anwalts	WEITERES VORGEHEN	siehe Mitteilung über die Übermittlung des internationalen Recherchenberichts (Formblatt PCT/ISA/220) sowie, soweit zutreffend, nachstehender Punkt 5
C 1974 PCT		
Internationales Aktenzeichen	Internationales Anmeldedatum *(Tag/Monat/Jahr)*	(Frühestes) Prioritätsdatum *(Tag/Monat/Jahr)*
PCT/EP 98/04810	31/07/1998	01/08/1997
Anmelder		
MAX-PLANCK-GESELLSCHAFT ZUR FORDERUNG DER et al.		

Dieser internationale Recherchenbericht wurde von der Internationalen Recherchenbehörde erstellt und wird dem Anmelder gemäß Artikel 18 übermittelt. Eine Kopie wird dem Internationalen Büro übermittelt.

Dieser internationale Recherchenbericht umfaßt insgesamt 3 Blätter.

[X] Darüber hinaus liegt ihm jeweils eine Kopie der in diesem Bericht genannten Unterlagen zum Stand der Technik bei.

1. **Grundlage des Berichts**

   a. Hinsichtlich der **Sprache** ist die internationale Recherche auf der Grundlage der internationalen Anmeldung in der Sprache durchgeführt worden, in der sie eingereicht wurde, sofern unter diesem Punkt nichts anderes angegeben ist.

   [ ] Die internationale Recherche ist auf der Grundlage einer bei der Behörde eingereichten Übersetzung der internationalen Anmeldung (Regel 23.1 b)) durchgeführt worden.

   b. Hinsichtlich der in der internationalen Anmeldung offenbarten **Nucleotid- und/oder Aminosäuresequenz** ist die internationale Recherche auf der Grundlage des Sequenzprotokolls durchgeführt worden, das

   [X] in der internationalen Anmeldung in Schriftlicher Form enthalten ist.

   [ ] zusammen mit der internationalen Anmeldung in computerlesbarer Form eingereicht worden ist.

   [ ] bei der Behörde nachträglich in schriftlicher Form eingereicht worden ist.

   [X] bei der Behörde nachträglich in computerlesbarer Form eingereicht worden ist.

   [X] Die Erklärung, daß das nachträglich eingereichte schriftliche Sequenzprotokoll nicht über den Offenbarungsgehalt der internationalen Anmeldung im Anmeldezeitpunkt hinausgeht, wurde vorgelegt.

   [X] Die Erklärung, daß die in computerlesbarer Form erfaßten Informationen dem schriftlichen Sequenzprotokoll entsprechen, wurde vorgelegt.

2. [ ] **Bestimmte Ansprüche haben sich als nicht recherchierbar erwiesen** (siehe Feld I).

3. [ ] **Mangelnde Einheitlichkeit der Erfindung** (siehe Feld II).

4. Hinsichtlich der **Bezeichnung der Erfindung**

   [X] wird der vom Anmelder eingereichte Wortlaut genehmigt.

   [ ] wurde der Wortlaut von der Behörde wie folgt festgesetzt:

5. Hinsichtlich der **Zusammenfassung**

   [X] wird der vom Anmelder eingereichte Wortlaut genehmigt.

   [ ] wurde der Wortlaut nach Regel 38.2b) in der in Feld III angegebenen Fassung von der Behörde festgesetzt. Der Anmelder kann der Behörde innerhalb eines Monats nach dem Datum der Absendung dieses internationalen Recherchenberichts eine Stellungnahme vorlegen.

6. Folgende Abbildung der **Zeichnungen** ist mit der Zusammenfassung zu veröffentlichen: Abb. Nr. 7

   [ ] wie vom Anmelder vorgeschlagen

   [ ] keine der Abb.

   [X] weil der Anmelder selbst keine Abbildung vorgeschlagen hat.

   [ ] weil diese Abbildung die Erfindung besser kennzeichnet.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 98/04810

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/68 A61K35/00 A61K38/00 A61K39/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 206 302 A (CIBA GEIGY AG) 30 December 1986 see page 3 - page 4	1-3,5, 10-12
A,P	EP 0 854 364 A (NARANG HARASH KUMAR) 22 July 1998 see claims; examples	1
A	US 5 234 814 A (CARD JOHN P ET AL) 10 August 1993 see column 4 - column 7; example 3	1
A	WO 96 12544 A (GEN HOSPITAL CORP ;TANZI RUDOLPH E (US); BUSH ASHLEY I (US); MOIR) 2 May 1996 see examples	1

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 April 1999	18/05/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreno, C

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TATEISHI, J. ET AL: "Removal of causative agent of Creutzfeldt-Jakob disease (CJD) through membrane filtration method." MEMBRANE, vol. 18, no. 6, 1993, pages 357-362, XP002101228 see the whole document	1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 98/04810

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0206302 A	30-12-1986	AU 5915186 A	08-01-1987
		DK 297086 A	26-12-1986
		FI 862595 A	26-12-1986
		GR 861603 A	20-10-1986
		JP 62002161 A	08-01-1987
		US 4782014 A	01-11-1988
EP 0854364 A	22-07-1998	NONE	
US 5234814 A	10-08-1993	AU 627046 B	13-08-1992
		AU 5817390 A	07-01-1991
		CA 2017832 A	01-12-1990
		EP 0474765 A	18-03-1992
		JP 4505865 T	15-10-1992
		WO 9015331 A	13-12-1990
WO 9612544 A	02-05-1996	AU 8083094 A	15-05-1996

VOSSIUS & PARTNER

Patentanwälte

Vossius & Partner POB 86 07 67 81634 München Germany

To the

European Patent Office

Munich

PATENTANWÄLTE
EUROPEAN PATENT ATTORNEYS
EUROPEAN TRADEMARK ATTORNEYS
Dr. VOLKER VOSSIUS, Dipl.-Chem.
(bis 1992; danach in anderer Kanzlei)
Dr. PAUL TAUCHNER, Dipl.-Chem.
Dr. DIETER HEUNEMANN, Dipl.-Phys.
Dr. PETER A. RAUH, Dipl.-Chem.
Dr. GERHARD HERMANN, Dipl.-Phys.
JOSEF SCHMIDT, Dipl.-Ing.
Dr. HANS-RAINER JAENICHEN, Dipl.-Biol.
Dr. ALEXA VON UEXKÜLL, M.Sc.
Dr. RUDOLF WEINBERGER, Dipl.-Chem.
Dr. WOLFGANG BUBLAK, Dipl.-Chem.
AXEL STELLBRINK, Dipl.-Ing.
Dr. JOACHIM WACHENFELD, (Biol.)
EUROPEAN PATENT ATTORNEYS
Dr. RENATE BARTH, Dipl.-Chem.
Dr. URSULA ENGLBRECHT, Dipl.-Chem.
RECHTSANWÄLTE
HELGA TREMMEL
BARBARA GUGGENMOS, Dipl.-Chem.

SIEBERTSTRASSE 4
81675 MÜNCHEN
GERMANY
TELEFON: +49-89-41 30 40
FAX: +49-89-41 30 41 11 (G3/G4)
+49-89-41 30 44 00 (G3)
(Marken - Trademarks)
E-MAIL: info@vossiusandpartner.com

Application No. PCT/EP98/04810
Max-Planck-Gesellschaft zu Förderung
der Wissenschaften e.V.
Our Ref.: C 1974 PCT

October 5, 1999
Wa/sg

This is in reply to the Written Opinion issued by the European Patent Office on July 27, 1999 in the above-referenced case.

1. Unity of the Invention (Rule 13.1 PCT)

Applicant holds the view that the inhibitor of claim 21 and the subject matter of claims dependent on claim 21 form a common inventive concept with the remainder of the claims since said inhibitor is a product that is identified (only) by the method of claim 13 et seq. In this regard, we wish the Examiner to note that we do not agree with the decision of the Technical Board in the case T 020/94 of November 4, 1999; see headnote 2.

Nevertheless, if the Examiner maintains his unity objections, applicant reserves the right to file any subject matter to be excised from the application in one or more divisional applications.

2. Clarity (Art. 6 PCT)

We do not concur with the Examiner's view that claim 1 contravenes the requirements of Art. 6 PCT (section VIII of the Written Opinion). Rather, the method of claim 1 requires two steps which can clearly be performed as such by the person skilled in the art and thus constitute technical features. The second step ("detecting whether said fibrils or aggregates are retained on said filter") teaches the person skilled in the art that a detection step is required in order to complete the method of the invention and arrive at a desired result. On the basis of his common general knowledge, the person skilled in the art is in the position to choose from various established methods to effect detection of bound material. An example of such an established method is provided on the bottom of page 3 of the application as filed.

In view of the above, we believe that step (b) of claim 1 is not only a result to be achieved, but is read by the person skilled in the art as a technical feature that can immediately be reduced to practice using conventional technology.

3. Novelty (Art. 33(2) PCT)

We respectfully submit that we cannot concur with the Examiner's view on novelty of the claimed invention either (section V of the Written Opinion). EP-A 0 206 302 (D1) teaches a method of immunologically analyzing serum amyloid A protein (SAA) and serum amyloid P-component (SAP). SAA is an α_1 globulin consisting of a single polypeptide chain with a molecular weight between 11,500 and 14,000 Daltons. SAP is a 9.5S α_1 glycoprotein of 235,000 Daltons (see D1, page 1, last paragraph). The selective binding of these proteins particularly to plastic surfaces, but also to filtered papers appears to depend on their interaction with bi-valent metal ions (D1, page 3, last paragraph). D1 is, however, completely silent on the possibility that detergents or urea-insoluble

amyloid-like fibrils or protein aggregates may be selectively retained on a filter. Consequently, the claimed subject matter is novel over the prior art.

4. Inventive Step (Art. 33(3) PCT)

We furthermore hold the view that the claimed subject matter cannot be derived from the disclosure content of D1 in an obvious manner. Particularly, we deny that the person skilled in the art, starting from the knowledge that certain serum proteins bind to solid carriers, would conclude without further ado that detergents or urea-insoluble amyloid-like fibrils or protein aggregates selectively bind to a filter material. For these reasons, we believe that the claimed subject matter is also inventive.

5. Requests

On the basis of the above, we request reconsideration of the Examiner's position such that a positive IPER be issued. Should any questions be outstanding and deemed necessary for discussion prior to the issuance of the IPER, the undersigned is prepared to discuss them via the telephone.

Dr. Joachim Wachenfeld
European Patent Attorney

PATENT COOPERATION TREATY

From the:
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:
VOSSIUS & PARTNER
Siebertstrasse 4
81675 München
ALLEMAGNE

EINGEGANGEN
Vossius & Partner
28. Juli 1999
Frist bearb.: 27.9.
27.8.99

PCT

WRITTEN OPINION

(PCT Rule 66)

Date of mailing *(day/month/year)*	27.07.99
Applicant's or agent's file reference: C 1974 PCT	**REPLY DUE** **within 2 month(s)** from the above date of mailing

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP98/04810	31/07/1998	01/08/1997

International Patent Classification (IPC) or both national classification and IPC
G01N33/60

Applicant
MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG ... et al.

1. This written opinion is the **first** drawn up by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

I	☒	Basis of the opinion
II	☐	Priority
III	☒	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain document cited
VII	☐	Certain defects in the international application
VIII	☒	Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also: For an additional opportunity to submit amendments, see Rule 66.4. For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis. For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 01/12/1999.

Name and mailing address of the international preliminary examining authority:
European Patent Office
D-80298 Munich
Tel. (+49-89) 2399-0 Tx: 523656 epmu d
Fax: (+49-89) 2399-4465

Authorized officer / Examiner
Wieser, M

Formalities officer (incl. extension of time limits)
Danti, B
Telephone No. (+49-89) 2399 8161

I. Basis of th opinion

1. This opinion has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".*):

Description, pages:

1-38 as originally filed

Claims, No.:

1-25 as originally filed

Drawings, sheets:

1/12-12/12 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. This opinion has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been and will not be examined in respect of:

☐ the entire international application,

☒ claims Nos. 21-25,

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. 21-25 are so unclear that no meaningful opinion could be formed (*specify*):

see separate sheet

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-3,5,10-12 NO
Inventive step (IS)	Claims	1-12 NO
Industrial applicability (IA)	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Section III:

The chemical products according to claims 21-25 are not defined in a way which allows to unambiguously identify them. These claims are not clear and do not meet the requirements of Article 6 PCT. A meaningful examination of novelty and inventive step is not possible.

Section V:

The subject-matter of claims 1-3,5 and 10-12 is anticipated by the disclosure in EP-A-0 206 302 (see claims and pages 3-4). These claims are not novel and do not meet the requirements of Article 33(2) PCT.

The subject-matter of claims 4 and 6-9 is not based on an inventive concept per se contrary to the requirements of Article 33(3) PCT.

Section VIII:

1. The subject-matter of independent claim 1 is not clear (Article 6 PCT).

   Article 6 PCT has to be interpreted as meaning not only that an independent claim must be comprehensible from a technical point of view but also that it must define clearly the object of the invention, that is to say indicate all the essential features thereof.

   At present claim 1 does not give any technical feature that would allow a skilled person to carry out the claimed detection method. The claim consists only of a statement describing the result to be achieved ("detecting whether said fibrils or aggregates are retained on said filter"). The information that the substances to be detected (claims 2-5) are indicative of a disease is unable to define a method for their detection.

2. Besides the objection raised in Section III above, the subject-matter of claims 21-25, does not seem to be so linked with the subject-matter of claims 1-20 as to form a single general inventive concept (Rule 13.1 PCT).

VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT
AUF DEM GEBIET DES PATENTWESENS

PCT

INTERNATIONALER RECHERCHENBERICHT

(Artikel 18 sowie Regeln 43 und 44 PCT)

Aktenzeichen des Anmelders oder Anwalts C 1974 PCT	**WEITERES VORGEHEN**	siehe Mitteilung über die Übermittlung des internationalen Recherchenberichts (Formblatt PCT/ISA/220) sowie, soweit zutreffend, nachstehender Punkt 5.
Internationales Aktenzeichen PCT/EP 98/ 04810	Internationales Anmeldedatum *(Tag/Monat/Jahr)* 31/07/1998	(Frühestes) Prioritätsdatum *(Tag/Monat/Jahr)* 01/08/1997
Anmelder MAX-PLANCK-GESELLSCHAFT ZUR FORDERUNG DER et al.		

Dieser internationale Recherchenbericht wurde von der Internationalen Recherchenbehörde erstellt und wird dem Anmelder gemäß Artikel 18 übermittelt. Eine Kopie wird dem Internationalen Büro übermittelt.

Dieser internationale Recherchenbericht umfaßt insgesamt 3 Blätter.

[X] Darüber hinaus liegt ihm jeweils eine Kopie der in diesem Bericht genannten Unterlagen zum Stand der Technik bei.

1. **Grundlage des Berichts**

   a. Hinsichtlich der **Sprache** ist die internationale Recherche auf der Grundlage der internationalen Anmeldung in der Sprache durchgeführt worden, in der sie eingereicht wurde, sofern unter diesem Punkt nichts anderes angegeben ist.

   [ ] Die internationale Recherche ist auf der Grundlage einer bei der Behörde eingereichten Übersetzung der internationalen Anmeldung (Regel 23.1 b)) durchgeführt worden.

   b. Hinsichtlich der in der internationalen Anmeldung offenbarten **Nucleotid- und/oder Aminosäuresequenz** ist die internationale Recherche auf der Grundlage des Sequenzprotokolls durchgeführt worden, das

   [X] in der internationalen Anmeldung in Schriftlicher Form enthalten ist.

   [ ] zusammen mit der internationalen Anmeldung in computerlesbarer Form eingereicht worden ist.

   [ ] bei der Behörde nachträglich in schriftlicher Form eingereicht worden ist.

   [X] bei der Behörde nachträglich in computerlesbarer Form eingereicht worden ist.

   [X] Die Erklärung, daß das nachträglich eingereichte schriftliche Sequenzprotokoll nicht über den Offenbarungsgehalt der internationalen Anmeldung im Anmeldezeitpunkt hinausgeht, wurde vorgelegt.

   [X] Die Erklärung, daß die in computerlesbarer Form erfaßten Informationen dem schriftlichen Sequenzprotokoll entsprechen, wurde vorgelegt.

2. [ ] **Bestimmte Ansprüche haben sich als nicht recherchierbar erwiesen** (siehe Feld I).

3. [ ] **Mangelnde Einheitlichkeit der Erfindung** (siehe Feld II).

4. Hinsichtlich der **Bezeichnung der Erfindung**

   [X] wird der vom Anmelder eingereichte Wortlaut genehmigt.

   [ ] wurde der Wortlaut von der Behörde wie folgt festgesetzt:

5. Hinsichtlich der **Zusammenfassung**

   [X] wird der vom Anmelder eingereichte Wortlaut genehmigt.

   [ ] wurde der Wortlaut nach Regel 38.2b) in der in Feld III angegebenen Fassung von der Behörde festgesetzt. Der Anmelder kann der Behörde innerhalb eines Monats nach dem Datum der Absendung dieses internationalen Recherchenberichts eine Stellungnahme vorlegen.

6. Folgende Abbildung der **Zeichnungen** ist mit der Zusammenfassung zu veröffentlichen: Abb. Nr. 7

   [ ] wie vom Anmelder vorgeschlagen [ ] keine der Abb.

   [X] weil der Anmelder selbst keine Abbildung vorgeschlagen hat.

   [ ] weil diese Abbildung die Erfindung besser kennzeichnet.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 98/04810

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/68 A61K35/00 A61K38/00 A61K39/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 206 302 A (CIBA GEIGY AG) 30 December 1986 see page 3 - page 4	1-3,5, 10-12
A,P	EP 0 854 364 A (NARANG HARASH KUMAR) 22 July 1998 see claims; examples	1
A	US 5 234 814 A (CARD JOHN P ET AL) 10 August 1993 see column 4 - column 7; example 3	1
A	WO 96 12544 A (GEN HOSPITAL CORP ;TANZI RUDOLPH E (US); BUSH ASHLEY I (US); MOIR) 2 May 1996 see examples	1

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 April 1999	18/05/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreno, C

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	TATEISHI, J. ET AL: "Removal of causative agent of Creutzfeldt-Jakob disease (CJD) through membrane filtration method." MEMBRANE, vol. 18, no. 6, 1993, pages 357-362, XP002101228 see the whole document	1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 98/04810

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0206302 A	30-12-1986	AU 5915186 A	08-01-1987
		DK 297086 A	26-12-1986
		FI 862595 A	26-12-1986
		GR 861603 A	20-10-1986
		JP 62002161 A	08-01-1987
		US 4782014 A	01-11-1988
EP 0854364 A	22-07-1998	NONE	
US 5234814 A	10-08-1993	AU 627046 B	13-08-1992
		AU 5817390 A	07-01-1991
		CA 2017832 A	01-12-1990
		EP 0474765 A	18-03-1992
		JP 4505865 T	15-10-1992
		WO 9015331 A	13-12-1990
WO 9612544 A	02-05-1996	AU 8083094 A	15-05-1996